MECHANISMS OF ADSORPTION OF *Actinomyces viscosus* TO HYDROXYAPATITE SURFACES

BY

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For Liza

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ABBREVIATIONS USED

CTMAC	Cetyltrimethylammonium chloride
EDTA	Ethylene diaminetetraacetic acid
FSB	Fibril-Sepharose bead
HA	Hydroxyapatite
IEP	Immunoelectrophoresis
IM	Intramuscular
IP	Intraperitoneal
IV	Intravenous
PAGE	Polyacrylamide gel electrophoresis
PAGE-SDS	Polyacrylamide gel electrophoresis in SDS
PAGE-urea	Polyacrylamide gel electrophoresis in urea
PBS	Phosphate buffered saline
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscopy
TC	Top common
TEM	Transmission electron microscopy
TSB	Tryptic soy broth
VA	Virulence associated

Abstract of Dissertation Presented to the Graduate Council of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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The purpose of this investigation was to examine the mechanism(s) by which the periodontopathogen *Actinomyces viscosus* strain T14V adhered to the tooth surface. This work was accomplished by utilizing an in vitro assay that has been shown to mimic the adsorption of bacteria to teeth in vivo. The problem was approached by comparing the in vitro adsorption of the virulent human isolate strain T14V with its avirulent variant, strain T14AV, to various saliva and/or serum pellicles formed on hydroxyapatite (HA) beads. In addition, the adherence receptor for this strain was identified, isolated, and partially characterized.

Cell numbers of strain T14V adsorbed to saliva-treated HA were greater than those of strain T14AV. Adsorption data

of strains T14V and T14AV to HA surfaces followed the Langmuir adsorption model as judged by high correlation coefficients obtained for both strains to most of the treated surfaces studied. The number of binding sites for strains T14V and T14AV cells to human saliva-treated HA and untreated HA was similar. The affinity of strain T14V for saliva--treated HA was tenfold greater than the affinity of strain T14AV for that surface.

To approximate the pellicle of the gingival crevice and margin and to determine whether adherence by strain T14V was to specific saliva or serum macromolecules, experimental pellicles were formed on HA by saliva/serum mixtures. The number of binding sites on the saliva/serum-treated HA remained the same as for the saliva-treated surface. Although the affinity of strain T14V cells for the saliva/serum HA surface remained generally the same as the affinity for the HA treated with saliva alone, the affinity of strain T14AV cells decreased further as the serum content increased. Strain T14V cell numbers adsorbed to serum-treated HA and albumin-treated HA were less than those adsorbed to saliva-treated HA, indicating that the adherence by strain T14V was to specific saliva receptors. In vivo results from streptomycin-resistant mutants of both strains T14V and T14AV confirmed in vitro results using saliva/serum pellicles.

Adsorption of strain T14AV to saliva-treated HA was effected by pH and ion concentration of the suspending buffer suggesting that hydrogen and ionic bonding played a role in

strain T14AV adsorption to this surface. In contrast, strain T14V adsorption to saliva-treated HA does not involve hydrogen bonding or electrostatic interactions. Pretreatment of strain T14V with proteolytic enzymes and heat inhibited adherence to saliva-treated HA, suggesting that the adherence receptor(s) on the cell surface of strain T14V was protein in nature.

Strain T14V possesses a fibrillar network on the cell surface and it has been hypothesized that these mediate bacterial adherence to surfaces. Fibril-mediated adherence of strain T14V cells to saliva-treated HA was studied. Fibrils were purified by ammonium sulfate precipitation and differential centrifugation from the crude supernatant of whole cells that were sheared by one passage through a French pressure cell. Purified fibrils and crude supernatant inhibited strain T14V adherence to saliva-treated HA to similar extents. However, anti-strain T14V serum, antifibril serum, and antifibril specific antibody completely abolished strain T14V adherence. The blocking immunoglobulin could be adsorbed from anti-T14V serum by strain T14V whole cells and purified fibrils. It was concluded that fibrils mediate adherence of strain T14V cell to saliva-treated HA. In addition, fibril preparations were shown to contain more than 95% protein and to be antigenically homogeneous by Laurell rocket immunoelectrophoresis.

INTRODUCTION

The purpose of this study was to investigate the mechanism(s) by which the periodontopathogen *Actinomyces viscosus* strain T14V adhered to the tooth surface. This work was accomplished by utilizing an in vitro assay that has been shown to mimic the adsorption of bacteria to teeth in vivo (14, 174). The problem was initially approached by comparing the in vitro adsorption of the virulent human isolate *A. viscosus* strain T14V with its avirulent variant, *A. viscosus* strain T14AV, to various saliva and/or serum pellicles formed on hydroxyapatite (HA) beads. The strain T14V adherence mechanism was further studied by identifying the receptor(s) on the bacterial cell surface mediating adherence. Finally, the bacterial cell surface receptor was isolated and characterized as to its composition and the role it assumes in adhering to specific salivary receptors.

Periodontal Disease

Periodontal disease is a general term that is used to encompass various diseases ranging from gingivitis to periodontitis. Both gingivitis and periodontitis begin at the dentogingival junction in the gingival crevice. Gingivitis refers to inflammation of gingival tissue whereas

peridontitis refers to the destruction and progressive disease that affects the gingiva, alveolar bone, and periodontal ligament. Gingivitis is an early stage of periodontitis; however, not all gingivitis cases progress to periodontitis.

Role of *Actinomyces viscosus* in Periodontal Disease

A. viscosus, a gram-positive rod, has been implicated in the etiology of gingivitis (73, 75, 102, 163) and periodontitis (74, 75). Colonization by this organism in germfree animals maintained on a soft, high carbohydrate diet results in plaque formation, root surface caries, and bone loss characteristic of periodontal disease (72, 74, 75). In humans it has been shown that accumulation of plaque on the tooth surface initiates gingivitis (100, 167). In addition, Listgarten demonstrated that gingivitis and periodontitis are characterized by a quantitative increase in the total mass of adherent organisms comprising plaque (98). Loesche and Syed (102, 163) have shown in humans that in experimental gingivitis, organisms from the *Actinomyces* species become the predominate cultivable organism as plaque accumulates. The predominate *Actinomyces* species isolated from subjects with nonbleeding gingivitis and bleeding gingivitis were *A. israelii* and *A. viscosus*, respectively. The increase in *Actinomyces* was accompanied by a decrease in *Streptococcus* species.

Immunological studies have shown that A. viscosus antigens penetrate the gingival tissue and elicit a T-cell response in various forms of periodontal disease (5, 63, 68, 89, 123, 150). Isolates of A. viscosus, A. naeslundii, Streptococcus sanguis, Bacteroides melaninogenicus, Veillonella, and Fusibacterium nucleatum were assayed for their ability to elicit a T-cell blastogenesis in subjects who had refrained from using oral hygiene (147). It was determined that only antigens from A. viscosus significantly increased the stimulation index as the gingivitis developed (147). The T-cell response returned to normal values when oral hygiene was reinstituted and gingivitis disappeared (147). In addition, it has been reported that the T-cell response to A. viscosus antigens decreased following treatment of naturally occurring gingivitis (130).

It had been proposed that gingivitis developed as a result of the nonspecific proliferation of the plaque flora (99, 151). Therefore, treatment of periodontal disease has been directed toward debridement of the dentogingival surfaces in order to eliminate large amounts of plaque (101). However experiments by Loesche and Syed (102, 163) suggest that if specific organisms are etiologically involved in gingivitis and other forms of periodontal disease, then antimicrobial treatment aimed at specific organisms might be effective (101).

General Consideration of Bacterial Attachment to Solid Surfaces

In the natural environment, many bacteria have a predilection for attaching to and colonizing surfaces. Solids immersed in seawater or freshwater become colonized by adherent microorganisms (178). In aquatic habitats, most of the permanent bacterial population is found on solid surfaces (27). In addition, bacteria have been observed to colonize the surfaces of sand grains (119), colloidal soil particles (106), algae (16), plant tissues (32, 144, 145), and other bacteria (71). The skin and mucous membranes of humans and animals are also heavily colonized by adherent indigenous bacteria (9, 59, 142). The initial step in the development of a bacterial infection entails attachment and colonization of the host tissue by the infecting organism (148). A variety of pathogenic bacteria, including beta-hemolytic streptococci (40), gonococci (173), Salmonella (164), Shigella (87), enteropathogenic Escherichia coli strains (149, 158), Vibrio cholerae (46), Clostridium species (2), Corynebacterium diphtheriae (7), etc., have been observed to attach to their host mucosal surfaces during natural or experimentally induced infections. In addition, dental pathogens such as Streptococcus mutans and Actinomyces species attach to and colonize the surfaces of teeth (59).

Even though surface colonization appears to be widespread in nature, only recently has the ecological significance of attachment or the mechanisms by which bacteria

adsorb to surfaces been investigated. ZoBell (178) was one of the first to appreciate the specificity of attachment of marine organisms to glass. He noted that of 96 isolates from sea water, 29 strains adhered strongly, 20 strains adhered variably, and 47 strains did not adhere. Zvyagintsev (179) further demonstrated that variation in adsorption could occur by strains within the same genus. The selectivity of bacterial adsorption to anion and certain exchange resins, cellulose fibers, charcoal, kaolin, bentonite, sand, and a variety of other adsorbents also has been shown (29).

Bacteria colonize different hosts and tissues with a high degree of specificity. For example, lactobacilli colonize the surface of nonsecreting keratinized stomach epithelial cells of rats and mice, but not the surface of secreting stomach epithelium (141). In addition, streptococci and other indigenous bacteria have been shown to selectively colonize the tongue, cheek, or tooth surfaces of the human mouth (52, 59, 81). Many pathogenic bacteria also display a restricted range of hosts or tissues to which they can attach and infect under natural conditions (49).

Studies of the ecology of indigenous bacteria in the human mouth provided the first convincing evidence that bacterial adherence is an ecological determinant of natural colonization (49, 59). The mouth contains several surfaces such as the teeth, tongue, buccal mucosa, palate and gingiva which are available for colonization. There is

evidence that these sites are selectively colonized by specific bacteria. For example, *Streptococcus salivarius* accounts for about half of the streptococcal populations found on the dorsal surface of the tongue; however, it is not found on teeth (52, 81). In contrast, *S. mutans* may comprise a significant proportion of the bacterial population that colonize the teeth, but its proportion on oral mucosal surfaces is generally low (59).

Several in vitro and in vivo assays have shown that indigenous streptococci and other bacteria selectively adsorb to specific oral surfaces, and that the experimentally derived affinities of these organisms directly correlate with their natural intraoral colonization (57, 59, 66, 94, 170, 171). Therefore, the bacterial cell surface apparently contains a highly developed recognition system capable of specifically interacting with specific host tissues. In addition, the correlation found between adherence and natural colonization strongly implies that adherence to host tissues is an important determinant of colonization for oral bacteria (49, 59).

Theoretical Considerations of Bacterial Attachment to Solid Surfaces

Bacterial adsorption to solid surfaces has been studied by several marine and soil microbiologists. ZoBell (178) and others (29, 108) noted that marine bacteria first became loosely associated with a surface and that firm attachment was a time dependent process, frequently associated with

the synthesis of holdfast material. The adsorption of bacteria to surfaces has been proposed to occur in two phases (107, 108). The initial phase of adsorption is considered to be reversible and the organisms are in a state of equilibrium. The bacteria are attracted to within 100 Å of the surface by Van der Waals forces; however, as the cell moves closer to the surface, the net negative charge on both the cell and the surface begin to exert a repulsive effect. The second phase of attachment involves an "irreversible" firm attachment of the bacteria to the surface through the interaction of macromolecules which effectively bridge the cell to the surface.

There are many important variables that could influence the sorptive behavior of bacteria. Growth culture medium, culture age, and bacterial cell concentration have been shown to effect adsorption properties of bacteria (29). In addition, environmental parameters such as the hydrogen ion concentration, the ionic strength, the incubation time, the incubation temperature and the degree of agitation are important considerations for bacterial adherence (29). Environmental parameters may affect the adherence of different bacteria in various ways. Adsorption of _Streptococcus salivarius_ and _S. pyogenes_ to human epithelial cells (40), of _Escherichia coli_ to urothelial cells (105), and of lactobacilli to rat stomach epithelium (160) do not differ significantly between pH 5 and 8. However, the adsorption of _S. mitior_ to saliva-treated HA shows a maximum at

pH 6 (96), and the adherence of gonococci to urothelial cells increases with increasing acidity (105). Therefore, it is evident that the optimal conditions for each bacterium-host tissue interaction must be delineated.

Bacterial Polymers Promoting Attachment to Surfaces

The ability of bacterial cells to interact with host tissue in a specific manner is thought to be the primary event by which most indigenous and pathogenic microorganisms initiate colonization of a host (59). Although little is known about the surface structures possessed by various bacteria, which apparently must interact with host tissue components to enable the microorganisms to become firmly attached and thereby resist elimination by host defense mechanisms (50), some information is becoming available. For example, fibrils, fimbriae, and pili have been implicated in the adsorption of many gram-negative (15, 19, 35, 36, 122, 129, 139, 140) and gram-positive (67, 84, 85, 177) microorganisms to host tissue. Pili have been shown to mediate adherence of gonococci to tissue culture cells (161), to erythrocytes (19), and to sperm cells (69) and of *Escherichia coli* to intestinal epithelial cells (122) and kidney cells (140). *Streptococcus salivarius* (60), *S. mitior* (95), and *S. pyogenes* (39) cells possess a "fuzzy coat" of thin, densely distributed surface fibrils that are morphologically distinct from pili. The interaction of

these fibrils with surface components of epithelial cells has been suggested from electron micrographs (39, 60). Treatment of these cells with trypsin removed the fibrils and greatly reduced the number of streptococci that attached to epithelial cells (39, 60). This suggested that the fibrils increased the strength of the adsorption bond between cell and surface. The S. pyogenes cell surface contains a type-specific M antigen that is a trypsin sensitive virulence factor (162). Further evidence that implicated the M antigen of S. pyogenes as an adherence factor was that M-negative mutants attached poorly and anti-M antibodies inhibited attachment (39). However, it has been shown that lipoteichoic acid extracted from heat-treated group A streptococci bound to epithelial cells and blocked the attachment of whole cells of the organism (10, 124). The observation that lipoteichoic acids binds to cell membranes suggests that these molecules may mediate bacterial adherence. However, the relative heat stability and trypsin resistance of lipoteichoic acid suggested that it was not the only factor promoting adherence of S. pyogenes to epithelial cell surfaces (10, 124).

In contrast to the observations with streptococci, it had been suggested that attachment of lactobacilli to chicken crop epithelium involved polysaccharide components (47). Evidence supporting this possibility are that attachment was not affected by heat or trypsin treatment, polyvalent concanvalin A agglutinated the adherent strains of

lactobacilli and treatment of these strains with monovalent concanavalin A inhibited attachment (47).

Theoretically, the chances for survival of a bacterial species in nature would be substantially increased if the organism had adapted to colonize many tissues and hosts during evolution. However, this does not appear to have occurred in view of the host and tissue tropisms of many indigenous and pathogenic bacteria. Alternatively, multiple surface components mediating adherence may be required for successful bacterial colonization of a host tissue rather than single surface receptors which might be easily altered by mutation. Evidence supporting this theory has come from studies involving the adherence of S. mutans. S. mutans cells specifically interacted with high molecular weight glycoproteins found in whole saliva (54) and in parotid saliva (43). Components of these salivary secretions have been shown to selectively adsorb to HA (43) and S. mutans cellular adherence occurred via interactions with these salivary components (59). Attachment of S. mutans cells to the salivary pellicle has been shown to involve a proteinaceous nonglucan receptor (25, 26, 86, 157). For example, S. mutans cells have been shown to attach to saliva-treated HA in the absence of sucrose (26). In addition, a glucosyltransferase defective mutant of S. mutans has been reported to attach to rodent teeth in vivo and saliva-treated HA in vitro (25). More recently, a lectin derived from Persea

americana seeds that is specific for protein side chains (118) has been shown to inhibit the attachment of S. mutans to saliva-treated HA (157). Accumulation of S. mutans on the tooth or HA surface was dependent upon the presence of glucan (48, 51, 114). Glucosyltransferase, a glucan synthesizing (114) and binding enzyme (51), can be located bound to the S. mutans surface where it synthesized glucan from sucrose (114) and acted as a receptor for additional glucan molecules located on other cells that resulted in cell to cell aggregation (51). Cell free glucosyltransferase molecules bound to the cell surface, bound glucan molecules and served as additional glucan-binding receptors (48). Furthermore, there appeared to be several additional cell surface receptors, different from glucosyltransferase, that bound glucan and allowed for additional mechanisms of S. mutans aggregation (113, 176). Therefore, S. mutans cells have many recognition molecules which are involved in its adherence and colonization.

Attachment to plant tissue by certain pathogenic plant bacteria also has been studied. Attachment of Agrobacterium tumefaciens to pinto bean leaves is blocked by lipopolysaccharides isolated from the organisms and therefore preventing the formation of tumors on the plant (97). Lipopolysaccharides isolated from avirulent Agrobacterium strains do not inhibit the attachment of the virulent organism illustrating the high degree of specificity. Infection of clover root hairs by Rhizobium trifolii is

initiated by specific attachment of the organism to a lectin made by the plant (4). This bacterium possesses a surface polysaccharide antigen which is serologically cross-reactive with a component on the root hairs (31). It has been suggested that polyvalent lectin joins the bacterium to the root hairs via these cross-reactive components (31).

A. viscosus may require specialized cell surface structures to promote adsorption and subsequent colonization on teeth, epithelial cells and other bacteria in plaque. Girard and Jacius (61) proposed that *Actinomyces* adsorption was mediated by the surface fibrils they observed in electron photomicrographs. Fibrils have been implicated in the coaggregation of *A. viscosus* (21, 116) or *A. naeslundii* (21, 37) with oral streptococci. In addition, *A. naeslundii* strains that possessed fibrils adhered to buccal epithelial cells whereas strains that had fibrils mechanically removed did not adhere to epithelial cells suggesting that fibrils mediated adherence of *A. naeslundii* to epithelial cells (41). Brecher et al. (14) have shown differences in virulence of *A. viscosus* strains T14V and T14AV in monoinfected germfree rats and conventional rats. Furthermore, these authors have shown that the prerequisite for virulence of strain T14V is its ability to attach to and colonize the teeth and gingival crevice of rodents, resulting in bone loss characteristic of periodontal disease (14). In this model, the failure of strain T14AV to result in alveolar bone loss was attributed to its colonization pattern.

Strain T14AV colonizes pits and fissures, but lacks the ability to colonize the gingival crevice region of teeth and therefore cannot induce periodontal disease in these laboratory animals (14).

A. viscosus strain T14V possesses a virulence-associated antigen(s) identified by immunoelectrophoresis (63, 132) or by immunodiffusion (23), which is not detectable in the avirulent variant A. viscosus strain T14AV. In addition, morphological and ultrastructural studies of strain T14V and T14AV have shown a characteristic fibril structure on the cell surface of strain T14V that is undetectable on the avirulent strain (14, 132). Cisar and co-workers have identified these fibrils isolated from cell walls as a virulence-associated antigen (22, 23). Because fibrils are numerous and predominant on the cell surface, it seems logical that they would be involved in adherence. However, a priori role of fibrils of A. viscosus strain T14V in the adherence to saliva-treated surfaces has not been demonstrated.

Host Tissue Receptors for Bacteria

The adsorption of bacteria is also influenced by the composition of the adsorbent surface (29, 50, 107). In vivo the teeth are covered by an acquired pellicle, formed of selectively adsorbed salivary components (42, 64, 110-112, 154). The presence of adsorbed salivary components on enamel or HA surfaces alters the composition and surface

characteristics of those surfaces (135) and clearly influences the selectivity of bacterial adsorption (24, 26, 53, 66, 95, 125, 127). For example, oral bacteria adsorbed differently to saliva-treated HA than to untreated HA (14, 24, 53, 66, 95, 174); the number of cells of some species which adsorbed is increased by the salivary pellicle, whereas the adsorption of other species is decreased. The role of the adsorbed salivary components as receptors on host tissues with which bacteria interact have not been well characterized. However, data are available which suggest that glycoproteins or glycolipids present on the teeth and epithelial cells may serve as the receptors for some species (175). Surface glycoproteins on human buccal epithelial cells are associated with adsorption of S. mitis, S. sanguis and S. salivarius (175). This was shown by masking the adherence receptors on the epithelial cells with antibody to specific blood group antigens or Concanavalin A. Blood group reactive salivary glycoproteins are present in the acquired pellicle on human teeth (152). Moreover, it has been shown that the same sugars which inhibit human erythrocyte hemagglutination by Leptotrichia buccalis cells also inhibit the organism's adsorption to saliva-treated enamel (80) suggesting that the molecules involved in both interactions possess similar binding determinants. In addition, oral Streptococcus and Actinomyces species selectively adsorbed blood group reactive glycoproteins from saliva and mucin (55). It has been reported that different specific

salivary glycoproteins interact with various related and non-related bacteria. Salivary constituents, particularly high molecular weight glycoproteins, bound to and aggregated a variety of oral species (65). The observation that salivary glycoproteins which aggregated strains of *S. mitis* were different from those which reacted with strains of *S. sanguis* provided evidence for specific interactions (76). In addition, Ørstavik (126) provided evidence that different strains within the same species may exhibit specificity for host sites. Saliva pre-adsorbed with one of two strains of *S. sanguis* was used to form a pellicle on enamel. It was observed that *S. sanguis* cells would not adhere to pellicles formed from saliva pre-adsorbed by the homologous strain; however, *S. sanguis* cells would adhere to pellicles formed from saliva pre-adsorbed by the heterologous strain.

Recent reports have suggested that the presence of adsorbed salivary components influenced the adsorption of oral bacteria by altering the strength of the adsorption bonds formed between the organism and the surface, by changing the number of receptor or adsorption sites that the surface provides for the organisms or by a combination of the two (24, 53). A mathematical model has been described which enables comparative estimates of these two parameters to be made (53, 82). The model essentially describes a Langmuir adsorption isotherm (90) which is often used in studies of molecular adsorption. Gibbons, Moreno and Spinell (53) found that the model adequately described the

adsorption of a *S. mitior* to untreated and to saliva-treated HA powder. Subsequently, the adsorption model has been utilized to describe the adsorption of several other streptococcal strains (1, 24) and two actinomyces strains (24) to HA and of *Rhizobium* sp. to root hairs (144, 145).

MATERIALS AND METHODS

Bacterial Strains

A. viscosus strains T14V and T14AV were kindly provided by B. F. Hammond, University of Pennsylvania, Philadelphia. *A. viscosus* strains T14VJ1 and T14AVT1 are laboratory-derived, streptomycin-resistant variants (200 μg/ml) of T14V and T14AV, respectively, and were isolated as previously described (171). All cultures were stored as lyophilized stocks or as multiple frozen stocks at -80°C in tryptic soy broth (Difco Laboratories, Detroit, Mich.) containing 20% glycerol.

Culture Conditions

Batch cultures were grown in tryptic soy broth with dextrose (TSB; Difco). Tritium-labeled cells of *A. viscosus* strains T14V and T14AV were prepared fresh from the frozen stocks for each experiment by growing the organisms in TSB containing 4 μCi of [^{3}H]thymidine per ml (sp. act. 3×10^{-3} cpm per cell; Schwarz/Mann, Orangeburg, N.Y.). Cultures were incubated under microaerophilic conditions (90% N_2, 10% CO_2) at 37°C in a Psycrotherm (New Brunswick Scientific Co., New Brunswick, N.J.) shaking incubator.

Electron Microscopy

HA beads with adsorbed bacterial cells were washed with three volumes of 0.05 M KCl containing 1 mM potassium phosphate (pH 7.3), 1 mM $CaCl_2$, and 0.1 mM $MgCl_2$ (buffered KCl) and placed in a vial and dehydrated through a graded acetone series. Beads were critical-point dried (Sorval, Newton, Conn.) and mounted on studs by sprinkling the beads onto double-coated tape (Scotch, No. 666). Studs were coated with gold palladium for 3 min at 10 mA on a Hummer II (Technics, Alexandria, Va.) plater and examined in a Novascan 30 (Zeiss, New York, N.Y.) scanning electron microscope (SEM).

For examination of unfixed whole cells or material extracted from cells, one drop of washed material was placed on carbon-stabilized Parlodion film. Excess material was removed, and 1 drop of 1% uranyl acetate in water was added. Excess stain was immediately removed, and the grids were examined at 60 kV in a Zeiss EM9 S-2 transmission electron microscope (TEM).

Acid Extraction of Whole Cells and Cellular Components

The weak acid chemical extraction of Lancefield and Perlmann (88) was used to solubilize antigens. Washed, freshly cultivated whole cells (0.25, [wet weight] per ml) or lyophilized extracted cellular components (0.25 mg [dry weight] per ml) of *A. viscosus* strains T14V or T14AV were suspended in 0.04 N HCl in 0.85% saline and placed in a

boiling water bath for 15 min. After cooling, the suspensions were titrated to neutrality by addition of 2.0 N NaOH in saline. Insoluble material was removed by centrifugation at 25,000 x g for 15 min and the supernatant lyophilized.

Antisera Preparation

Hyperimmune sera were prepared in adult New Zealand rabbits by a series of intravenous (IV) injections of *A. viscosus* T14V cells. Cells were grown to late exponential phase in TSB without dextrose (Difco) supplemented with 0.1% yeast extract (Difco) and 1% glucose, harvested, and washed with saline. Cells were suspended at 5 mg (wet weight) per ml in saline and placed in a boiling water bath for 15 min. Rabbits initially were injected with 0.1 ml of killed whole-cell suspension. Beginning with week 2 and continuing at weekly intervals, rabbits were injected with 1 ml of the cell suspension. Beginning with week 7, rabbits were bled once per week from the marginal ear vein. The sera was obtained and frozen at -30°C until subsequent use. No immune reactions were detected between antigen preparations and pre-immunization serum.

Rabbit immune serum to *A. viscosus* strain T14V purified surface fibrils was prepared by immunizing New Zealand rabbits with a series of intraperitoneal (IP) injections. Fibrils were suspended at 2 mg per ml in saline and dispersed by mild sonication (Kontes, Vineland, N.Y.). One milliliter of saline-fibril suspension and 1 ml of complete

Freund adjuvant (Difco) were mixed and injected IP twice a week for 4 weeks. Beginning with week 5, rabbits were bled from the marginal ear vein and reimmunized IP once a week.

Immune serum was prepared in a goat by a series of intramuscular (IM) and IV injections of strain T14V cells. Initially the goat was injected IM with 0.5 ml (10 mg) of killed whole cell suspension mixed with 0.5 ml of complete Freund adjuvant. A second 0.5 ml (10 mg) injection of whole cells without Freund adjuvant was given IM during week 2. At weeks 4, 6, 8, and 10, IV injections containing 1 mg of killed whole cells were given. Beginning with week 12 and continuing at weekly intervals, the goat was bled from the carotid artery and injected IV with a suspension containing 1 mg of killed whole cells.

Saliva Preparation

Whole paraffin-stimulated saliva (100 ml per collection) from one donor was collected in a container chilled in ice and heated at 56°C for 30 min to inactivate degradative enzymes (65). Heat-treated saliva was clarified by centrifugation at 12,000 x g for 10 min (24). Sodium azide was added at a final concentration of 0.04%, and this preparation was stored at -30°C until subsequent use.

Serum Preparation

Blood was collected (100 ml per collection) from the same saliva donor. The blood was allowed to clot overnight

at 4°C. Serum was then obtained after centrifugation of the clotted blood at 250 x g for 10 min. Sodium azide was added to the serum at a final concentration of 0.04%, and this preparation was stored at -30°C for subsequent use.

Bacterial Adsorption to Hydroxyapatite

A. viscosus organisms labeled with [^{3}H]thymidine were harvested from 16-h cultures by centrifugation at 1,300 x g for 10 min, washed twice and suspended in buffered KCl. Clumped organisms were dispersed with medium power 10-s pulses from a micro-ultrasonic cell disrupter (Kontes) between centrifugations and for three 10-s pulses before dilution to final concentrations. Final concentrations were determined from a plot of optical density versus concentration made in a Spectronic 20 spectrophotometer (Bausch and Lomb, Inc., Rochester, N.Y.). The plot was made from direct microscopic counts of organisms at various concentrations, using a hemacytometer (American Optical Corp., Buffalo, N.Y.).

Forty milligrams of hydroxyapatite beads (BDH Biochemicals Ltd., Poole, England) were washed twice and hydrated in distilled water, equilibrated in buffered KCl and treated with saliva, serum, saliva/serum mixtures, albumin and buffered KCl by the methods previously described by Clark et al. (75). After 90 min of incubation of HA beads with bacterial suspensions, the HA beads were allowed to settle from the mixture for 60 s. Aliquots, 100 μl each, were removed

from the supernatants, which contained unadsorbed organisms, and placed in vials containing 10 ml of Aqueous Counting Scintillant (Amersham/Searle, Arlington Heights, Ill.). Microscopic examination of bacterial suspensions revealed that cells were evenly dispersed during all phases of the experiment. The samples were allowed to equilibrate for 2 h in the dark at 4°C and were counted on a Searle Isocap 300 scintillation counter. Portions of known numbers of ^{3}H-labeled cells were counted in a similar manner so that counts per minute could be related to bacterial cell number. Control bacterial suspensions were incubated without HA beads and counted similarly to correct for cell loss due to adsorption to the tubes. Direct counts of bacterial adsorption to HA surfaces by SEM confirmed that this was a reasonably sensitive and reliable method for studying bacterial adherence to HA (26). Data are expressed as number of cells adsorbed per 40 mg HA, percent of cells adsorbed of the control (equation 1), or percent inhibition of the control (equation 2).

Equation 1:

$$\text{\% Adherence of Control} = \frac{\text{\# cells experimental adsorbed}}{\text{\# cells control adsorbed}} \times 100$$

Equation 2:

$$\% \text{ Inhibition of Control} = \frac{\text{\# cells control adsorbed} - \text{\# cell experimental adsorbed}}{\text{\# cells control adsorbed}} \times 100$$

All experiments were done in duplicate and repeated at least twice.

Calculations of Parameters for Bacterial Adsorption to HA Surfaces

Adsorption isotherms obtained by direct measurements were used to calculate the strength of the adsorption bond (i.e. affinity) and the number of binding sites, using the bacterial adsorption model described by Gibbons and co-workers (53). The adsorption model is described by the equation $C/Q = 1/KN + C/N$, where C is the concentration of free cells at equilibrium, N is the maximum number of binding or "receptor" sites, and Q is the total number of cells adsorbed per unit of adsorbent. At equilibrium, the strength of the adsorption bond between the bacterial cell and the adsorbent surface is described by parameter K (ml per cell). A plot of C/Q versus C yields a straight line if the experimental data are adequately described by the mathematical model.

Influence of Environment on Adherence

Various environmental parameters were altered within the adherence assay to determine the subsequent effect on bacterial adsorption to saliva-treated and untreated HA.

The buffered KCl parameters varied included pH (5.75-8.33), ionic strength (KCl M .001 M-2.0 M) and calcium concentration (0.0-10.0 mM). All other parameters within the buffered KCl were exactly the same except where stated.

To determine the influence of certain ions on the adsorption properties of both strain T14V and T14AV to HA, separate 1 mM potassium phosphate buffers (pH 7.3) were made to include: 1) 0.05 M KCl; 2) 1 mM $CaCl_2$; 3) 0.1 mM $MgCl_2$; 4) 0.05 M KCl, 1 mM $CaCl_2$; or 5) 0.05 M KCl, 0.1 mM $MgCl_2$. Both bacteria and HA beads were washed with the appropriate buffer prior to use in each experiment. In addition, controls for each experiment included treatment of the cells and HA with the normal buffered KCl.

The influence of various detergents on bacterial cell adherence to saliva-treated and untreated HA surfaces was examined. A non-ionic detergent, Tween-80 (Sigma Chemical Co., St. Louis, Mo.); an anionic detergent, sodium dodecyl sulfate (SDS; Sigma); and a cationic detergent, cetyl trimethylammonium chloride (CTMAC; Sigma) were included in various concentrations (0.001-1.0%) in the buffered KCl. Both strain T14V and T14AV cells were washed and suspended in these detergent containing buffers and used in the adherence assay. In another experiment, cells were washed once with buffered KCl containing 0.1% CTMAC or 0.01% SDS, washed twice in buffered KCl without detergent, resuspended in buffered KCl without detergent, and adherence to saliva-treated and untreated HA determined.

Enzyme Treatment of *A. viscosus* Cells

The influence of pretreating *A. viscosus* T14V or T14AV cells with various enzymes on their subsequent binding to saliva-treated HA was studied. Aliquots of cells (4×10^7 cells per ml) were treated with the following enzymes: 0.2% trypsin (type III, twice crystallized; Sigma Chemical Co., St. Louis, Mo.) in phosphate-buffered saline (PBS) adjusted to pH 8; 0.2 to 1.0% chymotrypsin (type II, Sigma) in PBS, pH 7.8; 0.2% papain (type II, twice crystallized, Sigma) in PBS, pH 6, containing 50 mM $CaCl_2$; 0.2% protease (type VIII, Sigma) in PBS adjusted to pH 7.8. The mixtures were incubated for 1 h at 37°C, except for the chymotrypsin which was at 25°C. After treatment, cells were collected by centrifugation and washed twice with 0.5 M NaCl and twice with buffered KCl, and inhibition of adherence was examined. Control cells were treated similarly with each buffer but without enzyme.

Periodate Treatment of *A. viscosus* Cells

Washed cells were suspended in 10 mM periodate in PBS at pH 6.5 and incubated for 12 h at 4°C. Cells were collected by centrifugation and washed twice with buffered KCl before the adherence assay to saliva-treated HA. Control cells were incubated in PBS without periodate.

Isolation of A. viscosus T14V Surface Fibrils

Washed whole cell suspensions of strain T14V were subjected to sonication, ballistic disintegration (Braun homogenization) and French press extraction according to the methods of Brown et al. (17). Whole cell and cell-wall free supernatants from the various extracts were examined for their ability to inhibit adherence as described below. Supernatants obtained by French press extraction were further purified as described below.

A modification of the method described by Buchanan (18) for the purification of gonococcal pili was used to isolate surface fibrils from A. viscosus strain T14V cells. Bacterial cells from 9 liters of TSB (100 g wet weight) were washed in 0.05 M potassium phosphate buffer (pH 7.2) and resuspended in the same buffer to form a thick paste. The cell paste was passaged once through a French pressure cell (American Instrument Co., Silver Spring, MD) at 10,000 lb/in^2. One passage resulted in less than 2% cell disruption, as measured by release of material adsorbing at 260 nm. The product was centifuged at 48,000 x g for 20 min to remove whole cells and cell walls. The crude supernatant was centrifuged for 24 h at 160,000 x g. The pellet was resuspended in 20 ml of 0.1 M tris (hydroxymethyl) aminomethane buffer (pH 7.5) and sonicated at full power for 1 min with a microultrasonic cell disrupter. After centrifugation for 10 min at 23,700 x g, the supernatant was mixed

with an equal volume of 20% ammonium sulfate and left at 4°C for 24 h. This solution was subsequently centrifuged for 15 min at 30,900 x g. The supernatant remaining after the first precipitation was reprecipitated by increasing the ammonium sulfate concentration to 30%. This procedure was continued up to an ammonium sulfate concentration of 60% in 10% gradations. Each resultant pellet was suspended in 10 ml of the appropriate concentration of ammonium sulfate, and vortexed for 1 min. After centrifugation for 15 min at 30,000 x g, the pellet was suspended in 10 ml of filtered distilled water, sonicated for 1 min at full power, mixed with the appropriate concentration of ammonium sulfate, and again left at 4°C for 24 h. The centrifugation process through the suspension in ammonium sulfate was repeated. The resulting pellet was suspended in filtered distilled water, dialyzed against four changes of distilled water, and lyophilized. Each purification step was monitored by TEM and Laurell rocket immunoelectrophoresis, as described below.

Cell Wall Preparation

Cell walls were kindly provided by David A. Brown, University of Florida. *A. viscosus* strain T14V washed cells were broken in a Braun cell homogenizer (Bronwill Scientific Inc., Rochester, N.Y., Model MSK) and walls were isolated and purified by the method of Bleiweis et al. (13).

Immunoelectrophoresis

The antigens present in the French press crude supernatant and the various ammonium sulfate precipitates obtained from the supernatant were detected by Laurell rocket immunoelectrophoresis (IEP, 91) utilizing the Osserman modification (83, 128) as described previously (132). To increase the solubility of the antigens, the material was suspended in 0.5% Triton X-100 (Sigma). In addition, 1.0% Triton X-100 was included in the agarose.

Polyacrylamide Gel Electrophoresis

Disc gel electrophoresis of the isolated surface fibrils was performed in 7.5% polyacrylamide gels containing 1% SDS (PAGE-SDS) according to the method previously described (77). Electrophoresis was performed at a constant current of 8.0 ma per tube until the tracking dye reached 5 mm from the bottom of the tube. Gels were stained with a 0.05% Coomassie blue R stain (Sigma) with 25% isopropanol and 10% glacial acetic acid overnight at 23°C, overnight a second time in a 0.025% Coomassie blue stain with 10% isopropanol and 10% glacial acetic acid, and a third night in 0.0125% Coomassie blue stain with 10% glacial acetic acid. Gels then were destained electrophoretically for 10 min in 7% acetic acid with a Canalco destainer II (Miles Lab., Inc., Elkhart, Ind.).

Column Chromatography

Isolated surface fibrils (1 mg/ml) were dissolved in 8 M urea and then 0.5 ml chromatographed over an ultrogel AcA 54 (LKB Instruments, Inc., Durham, N.C.) column (1.5 cm X 30 cm) that had been equilibrated with 8 M urea in 0.01 M tris (hydroxymethyl) aminomethane buffer (pH 8.4). The effluent was monitored by uv (280 nm), and 4 ml fractions were collected. Single peak fractions were pooled, extensively dialized against distilled water, and lyophilized. Peaks were then examined by PAGE and IEP methods previously described. Hen egg albumin, chymotrypsinogen A, and cytochrome C (Pierce Chemical Co., Rockford, Ill.) were utilized to standardize the column.

The above column system also was used with a 0.01 M potassium phosphate buffer (pH 7.2) solvent. Single peak fractions were pooled, dialyzed, and examined by TEM.

Chemical Analysis

Isolated fibrils were assayed for protein by the method of Lowry et al. (103), using bovine serum albumin (Sigma) as a standard. Carbohydrate, as measured by reducing sugars, was estimated by the phenol-sulfuric acid method (34), using dextran (Sigma) as a standard. Amino acid and amino sugar compositions of the purified fibrils were determined with an amino acid analyzer (Model JLC-5AH, JOEL USA, Inc., Cranford, N.J.). The fibril samples (2 mg) were hydrolyzed in 6 N HCl (2 ml), phenol (10 μl), and mercaptoethanol (5 μl) at 110°C

for 22 h in nitrogen-flushed, sealed vials. After lyophilization, the samples were suspended in distilled water (4 ml), filtered through glass wool, and analyzed.

Competitive Inhibition Assay

Various sugars and lyophilized preparations of purified fibrils or crude supernatant obtained by shearing whole cells as described previously were used in competitive inhibition assays with strain T14V cells and saliva-treated HA. Labeled strain T14V cells were suspended in buffered KCl at a concentration of 8×10^7 cells per ml. Equal volumes of buffered KCl containing various concentrations of sugars, fibrils, or crude supernatant were used to dilute the labeled cells to the standard working concentrations of 4×10^7 cells per ml. This mixture was assayed for adherence to saliva-treated HA. Bovine serum albumin (Sigma) and dextran (MW 18,400, Sigma) were used as controls to examine non-specific competitive inhibition. Additional controls consisted of strain T14V cells diluted with buffered KCl alone.

Antisera Adsorptions

In some experiments the goat and rabbit anti-strain T14V serum was adsorbed with strain T14V whole cells, purified fibrils, or cell walls as described below. Strain T14V cells were grown in TSB, harvested, washed twice and suspended in

the buffered KCl used in the adherence assay. Cell concentration was determined with a Spectronic 20 as previously described. A 5 ml aliquot of goat anti-T14V serum was adsorbed with 0.25-18.3 mg of fresh strain T14V cells for 60 min at 37°C. The adsorbent was removed by centrifugation at 48,300 x g for 10 min. The adsorbed serum was stored at -30°C for subsequent use. In order to relate cell number to dry weights, aliquots of known numbers of fresh washed whole cells used for the adsorption were lyophilized and weighed. An aliquot of goat anti-T14V serum was also adsorbed with various amounts of strain T14V cell walls for 60 min at 37°C. Cell walls were removed by centrifugation at 48,300 x g for 20 min. The adsorbed serum was stored at -30°C.

Goat anti-T14V serum was adsorbed with purified fibrils immobilized on Sepharose beads. Fibrils were convalently linked to CNBr activated Sepharose beads (4B, Sigma) by methods previously described (3, 28). With this procedure approximately 75% of a 10 mg sample of fibrils attached to 5 g of beads as measured by optical density at 280 nm. A 5 ml aliquot of serum was incubated with fibril-Sepharose beads (FSB) for 60 min at 37°C. The FSB adsorbent was removed by centrifugation at 225 x g for 1 min. The adsorbed serum was stored at -30°C for subsequent use. The FSB reagent was reactivated by washing twice with 0.05 M potassium phosphate buffer, pH 2.3, and twice with 0.5 M potassium phosphate buffer, pH 7.2. Goat anti-T14V serum adsorbed with

Sepharose beads without fibrils behaved similarly to non-adsorbed anti-T14V serum in all experiments.

Rabbit anti-T14V serum was also adsorbed with the FSB reagent as described above. Due to the high titer of the rabbit anti-T14V serum, the same 5 ml aliquot was adsorbed 3 times with the FSB reagent. After each adsorption, anti-fibril specific antibody was eluted from the immobilized fibrils by two 5 ml washes of 0.05 M potassium phosphate buffer, pH 2.3, and diluted immediately with 10 ml of 0.05 M potassium phosphate buffer, pH 7.2. The anti-fibril antibody from the 3 adsorptions was pooled, concentrated to 5 ml by ultrafiltration (PM-30 filter, Amicon Corp. Lexington, Mass.), and stored at -30°C.

Normal goat and rabbit sera (Grand Island Biologic Co., Grand Island, N.Y.) were adsorbed similarly by all the above adsorbents and used as controls.

Antisera Inhibition Assay

Adherence assays with various antibody preparations included in the cell-HA suspensions were done. Strain T14V cells suspended at 8×10^7 cells per ml were diluted to the standard cell concentration (4×10^7 cells per ml) with an equal volume of buffered KCl containing a 1:4 dilution of one of the antibody preparations or normal serum. Microscopic examination of the reaction mixtures revealed that significant clumping had not occurred at the cell concentrations and serum dilutions used. This serum dilution was

used in each antibody experiment unless indicated. Data are expressed as percent adherence of control cells.

In Vivo Experiments

To determine whether in vitro bacterial adsorption mimicked adsorption to human teeth in vivo, adsorption by _A. viscosus_ streptomycin-resistent strains T14VJ1 and T14AVT1 were compared for four human subjects. Both organisms were cultured overnight in TSB. Organisms were harvested by centrifugation, washed twice, and suspended in saline at a concentration of 4.0×10^9 cells per ml. Equal volumes of each cell suspension were mixed together, and dilutions of the mixture were plated in duplicate on triptic soy agar (BBL Microbiology Systems, Cockeysville, Md.) plates containing 200 µg of streptomycin per ml. These were incubated at 37°C. Proportions of colony-forming units of each strain in the mixture were determined by colony morphology and growth characteristics in TSB inoculated with one colony, and further confirmed by antigen extraction by the method of Lancefield and Perlmann (88). Antigens present in the extracts were detected by Laurell Rocket Osserman immunoelectrophoresis (91, 123) as modified by Powell et al. (132).

The six maxillary anterior teeth of four adult volunteers were cleaned by careful tooth brushing, and 1 ml of the actinomyces mixture was placed into the mouth of each subject. After 5 min, the mixture was expectorated, and the subjects thoroughly rinsed their mouths with water. The

buccal surfaces of three individual teeth on the right side were sampled by forceful rubbing with calgiswabs (Inolex Corp. Glenwood, Ill.) 15 min later (172). The three anterior teeth on the left side were sampled with calgiswabs after 4 h.

All swabs were immediately placed in 2 ml of saline containing 1% Trypticase (Difco), and the actinomyces were dispersed for 1 min by a Vortex mixer. Dilutions of the resulting suspensions were plated in duplicate on tryptic soy agar plates containing 200 μg of streptomycin per ml as described above. The relative proportions of colony-forming units of strain T14VJ1 to T14AVT1 recovered from teeth were multiplied by the reciprocal of their proportions in the mixture introduced into the mouth to reflect equal opportunity of attachment (96, 171).

RESULTS

Bacterial Adsorption to Treated and Untreated HA

The adsorption of *A. viscosus* strain T14V cells to saliva-treated HA was greater than to untreated HA (Table 1). This difference was not evident at low concentrations, but at initial concentrations of 4.0 X 10^7 cells per ml substantially more cells of strain T14V adsorbed to saliva-treated HA. In contrast, the adsorption of strain T14AV cells to saliva-treated HA was lower than to untreated HA at all initial cell concentrations tested. The number of strain T14V cells adsorbed to saliva-treated HA was 4- to 5-fold higher than the number of strain T14AV cells adsorbed to saliva-treated HA at initial concentrations of 4.0 X 10^7 cells per ml. The number of cells of both strain T14V and T14AV adsorbed to untreated HA was similar at all initial cell concentrations. Because the maximum difference in adherence to saliva-treated HA between strains T14V and T14AV occurred at 4.0 X 10^7 cells per ml and a saturated monolayer of cells was observed, 4.0 x 10^7 cells per ml was utilized to study the strain T14V adherence mechanism. Scanning electron micrographs (Fig. 1) of HA beads allowed visual observation of the differences in the adherence of

Table 1

Adsorption of *A. viscosus* to HA

HA pretreatment	Cells adsorbed[a]					
	Concn of *A. viscosus* T14V added (cells/ml)[b]			Concn of *A. viscosus* T14AV added (cells/ml)[b]		
	4×10^5	4×10^6	4×10^7	2×10^6	4×10^6	4×10^7
Untreated	4.4 ± 0.2	47.6 ± 1.2	200 ± 8	20.0 ± 0.8	35.2 ± 0.8	236 ± 12
Saliva	4.0 ± 0.1	43.2 ± 0.8	375 ± 12	8.0 ± 1.2	8.4 ± 2.0	86 ± 10
80% Saliva + 20% serum	4.0 ± 0.1	55.2 ± 0.4	400 ± 36	3.2 ± 1.2	4.8 ± 0.8	68 ± 4
20% Saliva + 80% serum	4.4 ± 3.6	48.8 ± 1.2	256 ± 4	0.8 ± 0.8	1.6 ± 1.6	24 ± 4
100% Serum			94 ± 10			
20% Saliva + 80% buffer			393 ± 1			
0.5% Albumin	1.6 ± 0.2	22.0 ± 3.6	120 ± 4			
7.0% Albumin	3.2 ± 1.2	16.0 ± 2.4	124 ± 4			

[a]Values indicate cells ($\times 10^5$) $\pm$ standard error per 40 mg of HA.

[b]Fixed concentrations indicated were added to tubes with total volume of 1.6 ml.

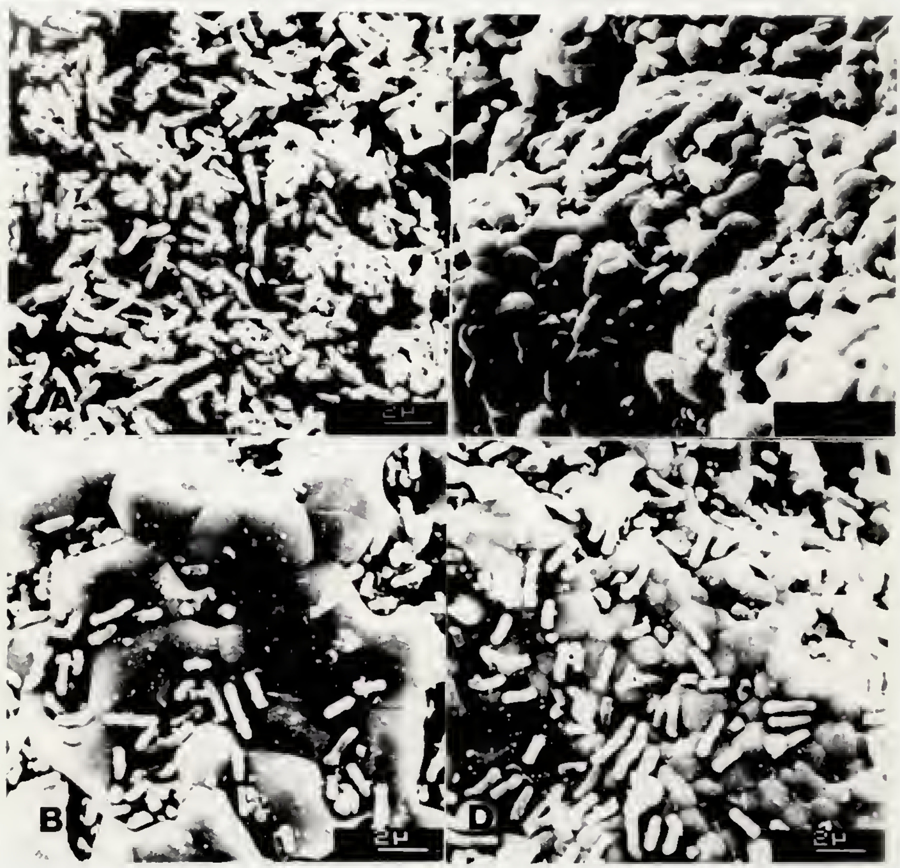

Figure 1. Scanning electron microscopy of A. viscosus to HA. Strain T14V to A) saliva-treated HA and B) untreated HA; Strain T14AV to C) saliva-treated HA and D) untreated HA. Bar in each micrograph represents 2 μm.

both strains T14V and T14AV to saliva-treated and untreated HA.

The pellicle in and around the gingival crevice may contain serum components, which might serve as binding sites, in addition to salivary constituents (58). Therefore, saliva/serum mixtures or serum alone were also used to form experimental pellicles. The addition of high concentrations of serum to saliva further reduced the number of strain T14AV cells which adsorbed to HA compared with HA treated with saliva alone (Table 1). The number of strain T14V cells adsorbed to HA treated with a mixture of 80% saliva/20% serum was similar to the number of cells adsorbed to HA treated with saliva alone. Moreover, HA treated with 20% saliva in buffered KCl adsorbed a similar number of strain T14V cells to that adsorbed by HA treated with saliva alone. However, HA treated with a 20% saliva/80% serum mixture adsorbed a fewer number of cells, suggesting that serum masks or blocks the salivary binding sites. When HA was treated with 100% serum, strain T14V adsorption was substantially reduced. These experiments were repeated with saliva and serum from different collection periods with identical results. Adsorption of strain T14V cells was inhibited to a similar extent by treatment of HA with either 0.5 or 7.0% albumin (Table 1). The number of strain T14V cells adsorbed to serum or albumin treated HA was dramatically less than the number of strain T14V cells adsorbed to saliva-treated HA,

gesting that these cells interact specifically with adsorbed salivary components rather than serum components.

Influence of Adsorbed Salivary Components to HA Surfaces on Adsorption Isotherms

The differences in adsorption of strain T14V and T14AV to treated and untreated HA were also apparent when data were plotted as isotherms (Fig. 2). Isotherms of strain T14V to saliva-treated and 80% saliva/20% serum-treated HA appeared nearly identical (Fig. 2A and B) and followed Langmuir kinetics. Adsorption of strain T14V to untreated or to 20% saliva/80% serum-treated HA and of strain T14AV to untreated or saliva-treated HA also follows Langmuir kinetics (Fig. 2A and B). Adsorption of strain T14V to albumin-treated HA (Fig. 2B) was dramatically less than its adsorption to any other treated or untreated HA surface. It was confirmed by SEM that adsorption of strain T14V to saliva-treated HA was limited to a monolayer over the range used to generate the isotherms. At initial concentrations equal to or less than 4.0×10^7 cells per ml, SEM observation demonstrated that monolayer adsorption had occurred (Fig. 3A and B). At concentrations of 4.0×10^8 or greater, cell to cell interactions and the formation of multiple layers of cells were observed (Fig. 3C and D).

Graphic plots of C/Q versus C, derived from the adsorption isotherms, generally resulted in straight lines for the various HA treatments (data not shown). The bacterial

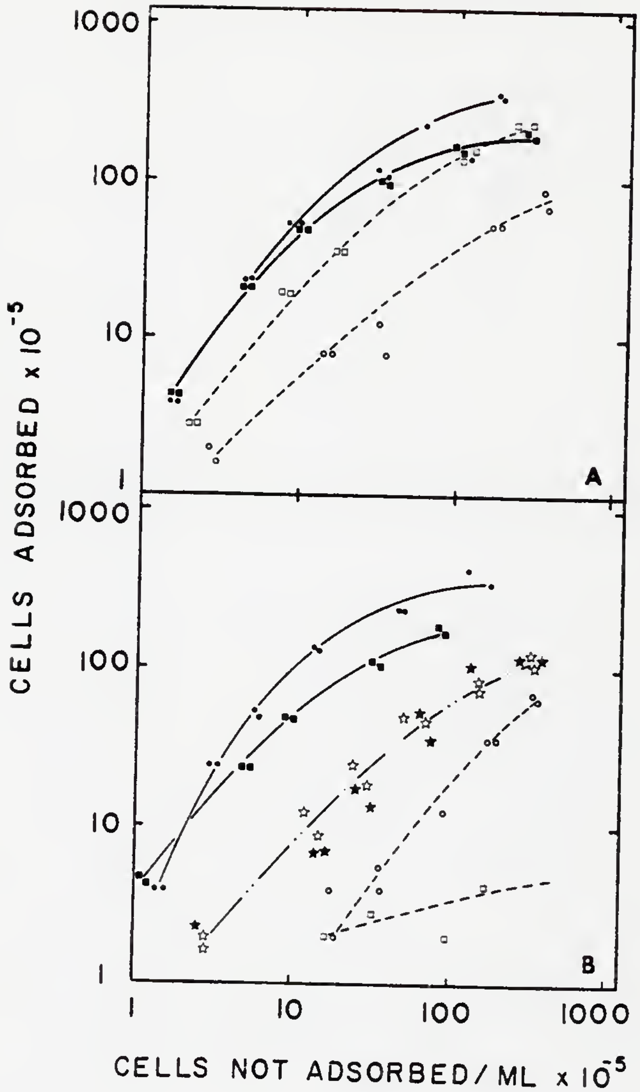

Figure 2. Adsorption isotherms of A. viscosus T14V and T14AV to saliva-treated, untreated, and saliva/serum-treated HA. (A) Strain T14V adsorption to saliva-treated (●) and untreated (■) HA. Strain T14AV adsorption to saliva-treated (○) and untreated (□) HA. (B) Strain T14V adsorption to 80% saliva/20% serum (●), 20% saliva/80% serum (■), and to 0.5% (☆) and 7.0% (★) albumin-treated HA. Strain T14AV adsorption to 80% saliva/20% serum (○) and 20% saliva/80% serum (□)-treated HA.

Figure 3. Scanning electron microscopy of various concentrations of A. viscosus T14V adsorbed to saliva-treated HA. (A) Initial concentration, 4.0 X 10^6 cells/ml; (B) initial concentration, 4.0 X 10^7 cells/ml; (C) initial concentration, 4.0 X 10^8 cells/ml; (D) initial concentration, 4.0 X 10^9 cells/ml. The bar represents 1 μm on all micrographs.

affinity for a surface as well as the number of binding sites available on the surface for that bacterium can be calculated from the slope and y-intercept of the line. The generally high correlation coefficients of experimentally derived data indicate that the previously described mathematical model delineates the adsorptive behavior of the organisms studied to a satisfactory degree (Table 2). This did not hold true when a high percentage of cells did not adsorb to the HA, as was observed for adsorption of strain T14AV to saliva/serum-treated HA (Table 2).

The calculated number of binding sites (parameter N) for both strains T14V and T14AV cells to saliva-treated HA were similar to those for untreated HA (Table 2). The strength of the bacterial adsorption bonds (parameter K) to untreated HA was similar for both strains T14V and T14AV. The affinity of strain T14V for saliva-treated HA was 10-fold greater than the affinity of strain T14AV for the same surface. The affinity of strain T14V for HA treated with saliva/serum mixtures was more than 100-fold greater than that calculated for strain T14AV cells to those surfaces.

Adsorption of *A. viscosus* to Human Teeth *In Vivo*

Higher cell numbers of strain T14VJ1 than strain T14AVT1 adsorbed to human teeth in vivo (Table 3). There was an average of 6.3-fold more cells of strain T14VJ1 recovered

Table 2

Estimates of Affinities and Adsorption Sites of *A. viscosus* T14V and T14AV on HA

	N^a		K^b		Correlation coefficients	
	T14V	T14AV	T14V	T14AV	T14V	T14AV
Untreated	2.4	3.4	2.0	1.0	0.97	0.97
Saliva	3.3	3.3	1.0	0.11	0.88	0.93
80% Saliva + 20% serum	5.0	12.0	2.0	0.016	0.96	0.67
20% Saliva + 80% serum	3.3	N^c	2.0	N	0.96	N
0.5% Albumin	1.0		1.0		0.85	

[a]Each value ($\times 10^7$) indicates number of adsorption sites per 40 mg of HA.

[b]Each value ($\times 10^{-7}$) indicates affinity constant in milliliters per cell.

[c]N, Not determinable due to low percentage of adsorption.

Table 3

Adsorption of *A. viscosus* T14VJ1 and T14AVT1 to Human Tooth Surfaces

Subject	Adsorption[a]			
	0.25 h		4.0 h	
	Strain T14VJ1	Strain T14AVT1	Strain T14VJ1	Strain T14AVT1
1	2.18 $\pm$ 0.17	0.47 $\pm$ 0.20	0.46 $\pm$ 0.24	0.098 $\pm$ 0.074
2	1.80 $\pm$ 0.20	0.50 $\pm$ 0.01	0.66 $\pm$ 0.24	0.050 $\pm$ 0.010
3	4.6 $\pm$ 2.0	0.66 $\pm$ 0.24	3.2 $\pm$ 1.8	0.24 $\pm$ 0.19
4	6.0 $\pm$ 3.0	0.06 $\pm$ 0.10	3.3 $\pm$ 1.7	0.43 $\pm$ 0.37

[a]Each value indicates number of cells ($\times 10^3$) $\pm$ standard error and is the mean of three teeth sampled in each subject, at the indicated times after organisms were introduced.

from all teeth as compared with strain T14AVT1 15 min after introduction into the mouth of a bacterial mixture containing equal amounts of organisms. An average of 10-fold more strain T14VJ1 cells than strain T14AVT1 cells were isolated from all tooth samples taken at 4 h. Therefore, during the 4-h time period, the numbers of avirulent strain T14AVT1 cells, which were adsorbed to the teeth, decreased to a greater extent than did the numbers adsorbed virulent strain T14VJ1 cells.

Influence of Ionic Environmental Parameters on *A. viscosus* Adsorption

The influence of the buffered KCl pH on adsorption of strains T14V and T14AV to saliva-treated and untreated HA is shown in Figure 4. Varying the pH of the buffered KCl did not dramatically influence strain T14V adsorption to saliva-treated HA. However, adsorption of strain T14AV to saliva-treated HA was enhanced by approximately 80% as the pH of the suspending buffer decreased from 7.0 to 5.7. Adsorption of both strains to untreated HA decreased slightly as the pH increased to 7.1. Varying the pH of the saliva (pH 6.0-8.0) prior to the formation of the pellicle on HA did not alter the adsorption of either strain to saliva-treated HA (data not shown).

Increasing the ionic strength of the buffered KCl, decreased the adsorption of strain T14V to both saliva-treated and untreated HA (Table 4). Adsorption of strain

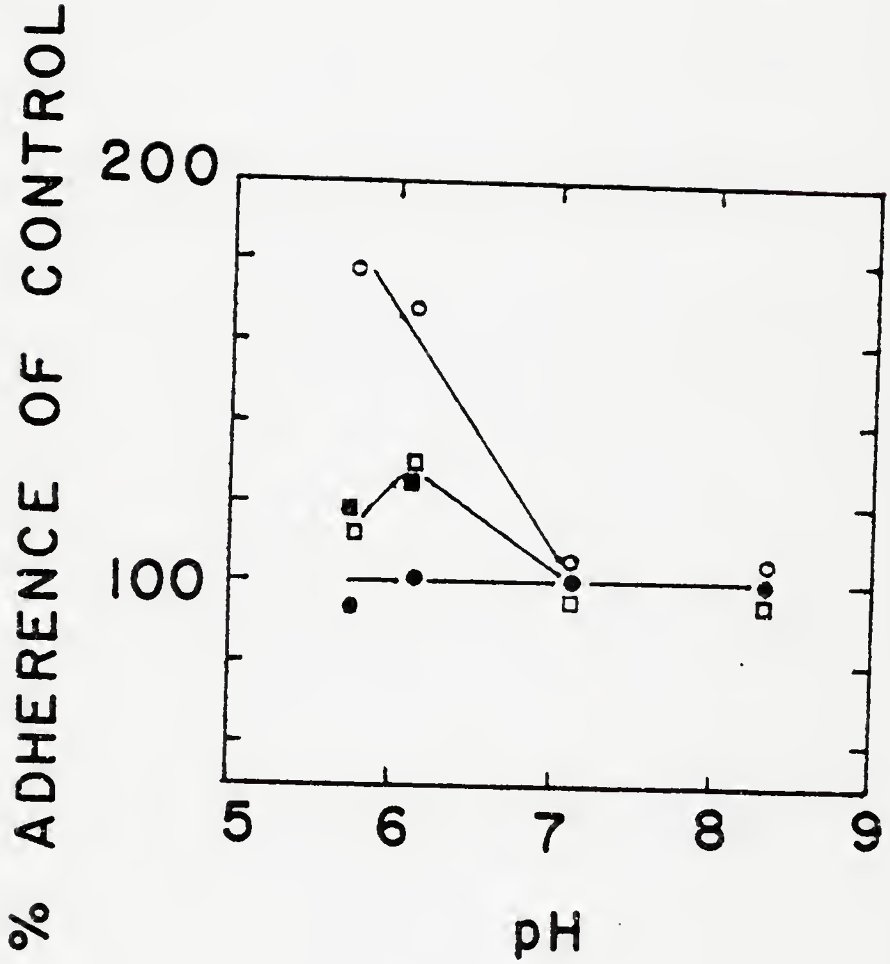

Figure 4. Influence of pH on adsorption of A. viscosus strain T14V to A) saliva-treated (●) and B) untreated HA (■) and of strain T14AV to C) saliva-treated (○) and D) untreated HA (□).

Table 4

Influence of Ionic Strength on Adsorption of *A. viscosus* to HA

	% Adherence of Control[a]			
	T14V		T14AV	
KCl(M)	Saliva-treated	Untreated	Saliva-treated	Untreated
0.001	150 ± 1[b]	167 ± 10	112 ± 13	63 ± 1
0.01	136 ± 1	160 ± 11	133 ± 7	102 ± 2
0.10	96 ± 8	109 ± 6	86 ± 10	103 ± 1
0.50	81 ± 8	96 ± 10	58 ± 11	119 ± 9
1.00	73 ± 4	52 ± 2	55 ± 7	103 ± 1
2.00	77 ± 5	57 ± 4	36 ± 2	76 ± 1

[a]Control is buffered KCl.

[b]Mean ± standard deviation.

T14AV to saliva-treated HA diminished as the KCl molarity increased. However, adsorption of strain T14AV to untreated HA was enhanced as the ionic strength increased up to 0.50 M, but then decreased when cells were suspended in higher salt buffers.

Adsorption of strain T14V to saliva-treated HA diminished slightly as the calcium concentration of the buffered KCl increased (Table 5). Strain T14AV adsorption to saliva-treated HA was greater when cells were suspended in 5×10^{-3} M calcium than at lower molar concentrations. Adsorption of both strains T14V and T14AV to untreated HA was not significantly altered by variations in the calcium concentration. Pretreatment of strain T14V and T14AV with 0.1 M ethylenediamine-tetraacetic acid at pH 8 had no effect on adherence in reaction mixtures containing PBS without Ca^{2+} or Mg^{2+}.

The effect of various ions on the adsorption of strain T14V to saliva-treated HA is shown in Table 6. The addition of K^{+}, Ca^{2+}, or Mg^{2+} singly or in any combination to the phosphate buffer significantly decreased the adsorption to a similar extent.

Strain T14V and T14AV adsorption to untreated HA increased when the cationic detergent CTMAC was included in cell-HA suspensions or when cells were pretreated with CTMAC (Table 7). Strain T14V adsorption to saliva-treated HA drastically decreased when CTMAC was included in the cell-HA suspensions whereas pretreatment of this strain with the

Table 5

Influence of Calcium Concentration on Adsorption of *A. viscosus* to HA

	% Adherence of Control[a]			
	T14V		T14AV	
Ca^{2+} (mM)	Saliva-treated	Untreated	Saliva-treated	Untreated
0.0	111 ± 6[b]	95 ± 3	ND[c]	ND
0.5	97 ± 5	92 ± 8	111 ± 10	77 ± 1
1.0	100 ± 1	100 ± 1	100 ± 3	100 ± 3
2.0	88 ± 1	94 ± 7	ND	ND
5.0	68 ± 5	84 ± 4	171 ± 25	94 ± 5

[a]Control is buffered KCl.

[b]Mean ± standard deviation.

[c]ND, not done.

Table 6

Influence of Ions on Adsorption of
A. viscosus T14V to Saliva-Treated HA

Ions included in buffer[a]	% Adherence of Control
None (Control)	100 ± 1[b]
50 mM KCl	60 ± 1
1 mM $CaCl_2$	64 ± 1
0.1 mM $MgCl_2$	59 ± 9
50 mM KCl, 1 mM $CaCl_2$	67 ± 1
50 mM KCl, 0.1 mM $MgCl_2$	57 ± 4

[a] 1 mM potassium phosphate buffer, pH 7.2.

[b] Mean ± standard deviation.

Table 7

Influence of Detergents on Adsorption of *A. viscosus* to HA

	% Adherence of Control			
	T14V		T14AV	
Cell treatment	Saliva-treated	Untreated	Saliva-treated	Untreated
Untreated (Control)	100 ± 1[a]	100 ± 1	100 ± 1	100 ± 1
CTMAC[b]	4 ± 2	184 ± 3	126 ± 5	154 ± 1
CTMAC-Buffer[c]	158 ± 1	236 ± 1	188 ± 7	126 ± 4
SDS[d]	140 ± 4	82 ± 4	107 ± 4	52 ± 8
SDS-Buffer[c]	128 ± 14	128 ± 1	118 ± 1	90 ± 4
Tween 80[e]	99 ± 4	105 ± 3	82 ± 1	89 ± 6

[a]Mean ± standard deviation.

[b]CTMAC, cells suspended in 0.10% cetyltrimethylammonium chloride.

[c]CTMAC-Buffer, SDS-Buffer, cells suspended in detergent, then washed with buffered KCl and resuspended in buffered KCl.

[d]SDS, cells suspended in 0.01% sodium dodecylsulfate.

[e]Tween 80, cells suspended in 1.0% Tween 80.

same detergent produced a marked increase in cell numbers adsorbed over control cell numbers. Strain T14AV adsorption to saliva-treated HA increased slightly in the presence of CTMAC and after pretreatment of cells with the detergent.

Strain T14V adsorption to saliva-treated HA increased when SDS was included in the cell-HA suspension or when cells were pretreated with the detergent (Table 7). Adsorption of strain T14V to untreated HA or of strain T14AV to saliva-treated HA was not influenced by the SDS treatments. Pretreatment of strain T14AV did not influence adsorption to untreated HA whereas SDS included in the cell-HA suspension decreased adsorption to that surface.

Neither strain T14V nor strain T14AV adsorption to saliva-treated or untreated HA was altered by the non-ionic detergent Tween-80 (Table 7).

Effect of Heat, Proteolytic Enzymes, and Periodate on Adherence of *A. viscosus* Cells to Saliva-Treated HA

Heating *A. viscosus* of either strain T14V or T14AV cells at 100°C for 15 min reduced their ability to adhere to saliva-treated HA (Table 8). However, adherence of both strains was not influenced by 60° or 80°C heat for 15 min. When examined by TEM (Fig. 5), strain T14V cells that had been heated to 60° or 80°C appeared similar to control cells. In contrast, strain T14V cells heated to 100°C lacked the surface fibrils present on the control cells.

Table 8

Effect of Various Pretreatments of *A. viscosus* Cells on Adherence to Saliva-Treated HA

Pretreatment	% Adherence of Control[a]	
	T14V	T14AV
None (Control)	100 ± 1[b]	100 ± 1
60°C, 15 min	100 ± 2	100 ± 1
80°C, 15 min	97 ± 1	100 ± 2
100°C, 15 min	29 ± 6	47 ± 6
0.2% Papain	178 ± 1	136 ± 3
0.2% Protease	8 ± 1	0 ± 2
0.2% Trypsin	29 ± 2	ND[c]
0.2% Chymotrypsin	68 ± 1	12 ± 5
0.4% Chymotrypsin	47 ± 2	ND
0.6% Chymotrypsin	29 ± 1	ND
1.0% Chymotrypsin	18 ± 1	ND
10 mM Periodate	53 ± 1	12 ± 2

[a]Control cells were treated separately for each enzyme.

[b]Mean ± standard deviation.

[c]ND, not done.

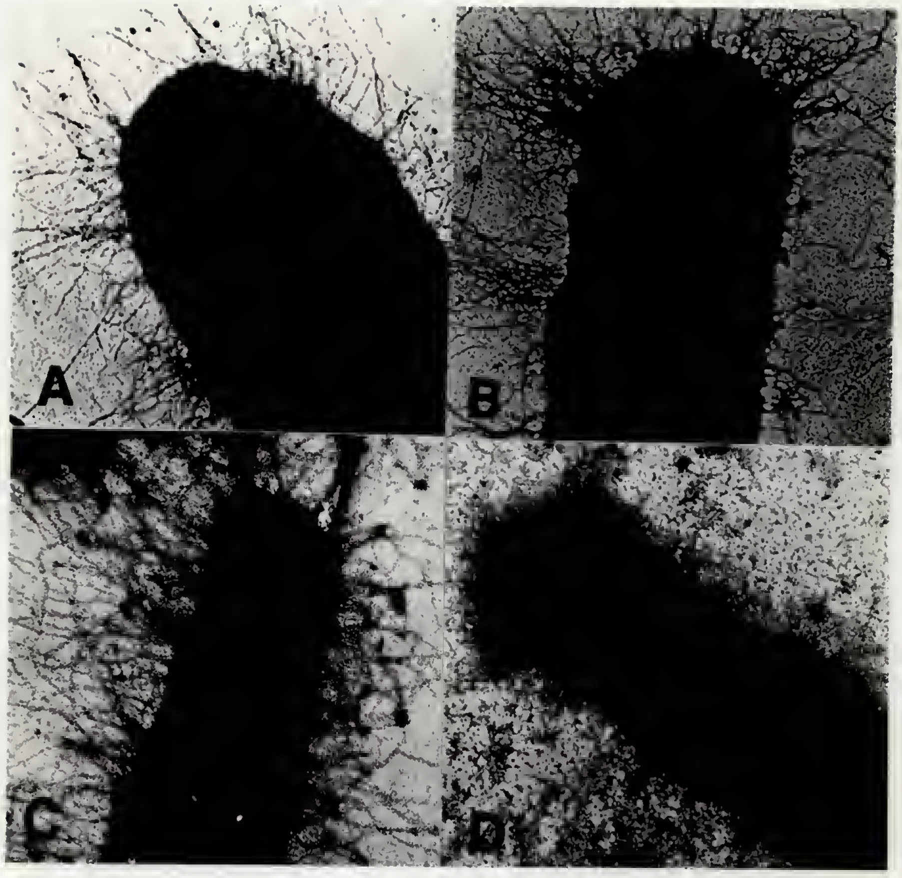

Figure 5. Transmission electron micrographs of uranyl-acetate stained preparations of heat-treated A. viscosus T14V. A) Control, 23°C, B) 60°C, C) 80°C, and D) 100°C.

Pretreatment of strain T14V or T14AV cells with protease, trypsin, and chymotrypsin inhibited their subsequent adherence to saliva-treated HA (Table 8). Pretreatment of strain T14V or T14AV cells with papain increased their adherence to saliva-treated HA, indicating that enzyme may remain bound or alter the cell surface and therefore promote adherence. Pretreatment of strain T14V with higher concentrations of chymotrypsin inhibited its adherence by as much as 82%, indicating that it also was sensitive to this enzyme, as was strain T14AV. Pretreatment of both bacterial strains with periodate inhibited adherence (Table 8). This suggests that carbohydrate moieties present on the bacterial cell surface could be associated with adherence. However, periodate could also cause protein alterations which could influence adherence. Electron microscopy of the enzyme- and periodate-treated strain T14V cells did not reveal any morphological changes from control cells (data not shown).

Competitive Inhibition of Adherence

Various sugars were included into the bacteria HA suspension to examine direct inhibition of strain T14V adherence to saliva-treated HA (Table 9). There was little, if any, inhibition by any of the various hexoses and discharides tested. Fructose, glucosamine and N-acetyl glucosamine did inhibit slightly at 0.1 M concentrations. However, when glucosamine and N-acetyl glucosamine concentrations were increased to 0.5 M, percent inhibition of

Table 9

Competitive Inhibition of *A. viscosus* T14V to Saliva-Treated HA *with Sugars*

Sugar	% Inhibition of Control
None (Control)	0 ± 1[a]
0.1 M Glucose	0 ± 1
0.1 M Galactose	6 ± 1
0.1 M Mannose	2 ± 1
0.1 M Sucrose	5 ± 1
0.1 M Maltose	0 ± 1
0.1 M Lactose	0 ± 1
0.1 M Rhamnose	7 ± 1
0.1 M Fructose	13 ± 1
0.1 M Galactosamine	0 ± 1
0.1 M Glucosamine	16 ± 2
0.5 M Glucosamine	20 ± 2
0.1 M N-acetylglucosamine	11 ± 1
0.5 M N-acetylglucosamine	23 ± 1

[a]Mean ± standard deviation.

strain T14V to saliva-treated HA did not increase significantly.

Crude supernatants obtained by sonication, Braun homogenization, or French press extraction of whole strain T14V cells were included into the bacterial cell-HA suspension to examine direct inhibition of strain T14V adherence to saliva-treated HA by cell-surface components released by those techniques (Table 10). At a concentration of 1 mg per ml, the French press extract of strain T14V inhibited adherence to a greater degree than did the extracts obtained by sonication or Braun homogenization. Therefore, the French press extract was studied further for the presence of a specific adherence inhibiting structure. An inhibiting component, isolated from the extract, was subsequently identified as strain T14V cell-surface fibrils.

Purified fibrils or crude French press supernatant were included in the bacterial cell-HA suspension to examine direct inhibition of strain T14V adherence to saliva-treated HA (Fig. 6). Increasing the amount of purified fibrils or crude supernatant inhibited adherence of whole cells up to a maximum of 30%. To assess the influence of nonspecific inhibition of adherence, bovine serum albumin and dextran were included in bacterial cell-HA suspensions in quantities similar to those tested for fibrils and crude supernatant. At the highest concentration of BSA or dextran examined (2 mg/ml), inhibition was approximately 10%. When the cell

Table 10

Competitive Inhibition of *A. viscosus* T14V to Saliva-Treated HA with Whole Cell Extracts

Extract (1 mg/ml)	% Inhibition of Control
Control	0 ± 2[a]
Sonic	16 ± 6
Braun Homogenization	17 ± 12
French Press	36 ± 1

[a]Mean ± standard deviation.

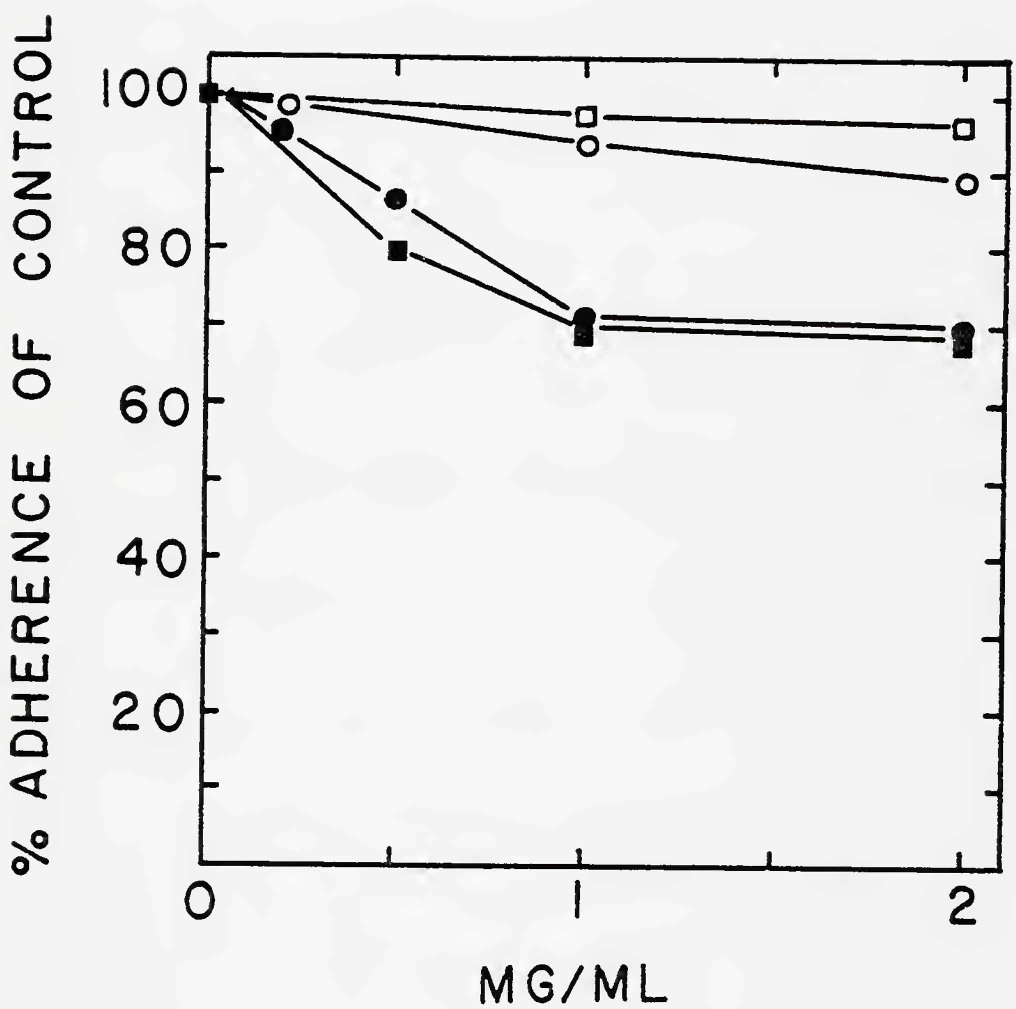

Figure 6. Competitive inhibition of A. viscosus T14V adherence to saliva-treated HA with French press crude supernatant (●), purified fibrils (■), bovine serum albumin (○), and dextran (□).

concentration was decreased to 4×10^5 cells per ml, fibrils (1 mg/ml) inhibited whole cell adherence by 47%.

Adherence Inhibition by Specific Antisera

Goat anti-T14V serum inhibited adherence of strain T14V to saliva-treated HA (Fig. 7). Adherence decreased as the concentration of antiserum in the bacterial cell-HA suspension increased. At antiserum dilutions equal to or less than 3:10, adherence was completely abolished. Normal goat serum did not diminish adherence of strain T14V cells.

Adsorption of Antiserum Inhibition Activity

The inhibition activity of the goat anti-T14V serum could be adsorbed completely with strain T14V cells or isolated fibrils and to a lesser degree with strain T14V cell walls (Table 11). Adherence inhibition activity was diminished in specific antisera as the number of strain T14V cells used to adsorb the serum increased (Fig. 8). However, adsorption of antisera with strain T14V cell walls did not reduce inhibition to the same extent as did whole cells. Adsorption of normal serum with whole cells or fibrils had little influence on adherence.

Adherence Inhibition with Antifibril Specific Antibody and Antiserum

Inhibition of strain T14V adherence to saliva-treated HA with rabbit anti-T14V serum was similar to goat anti-T14V

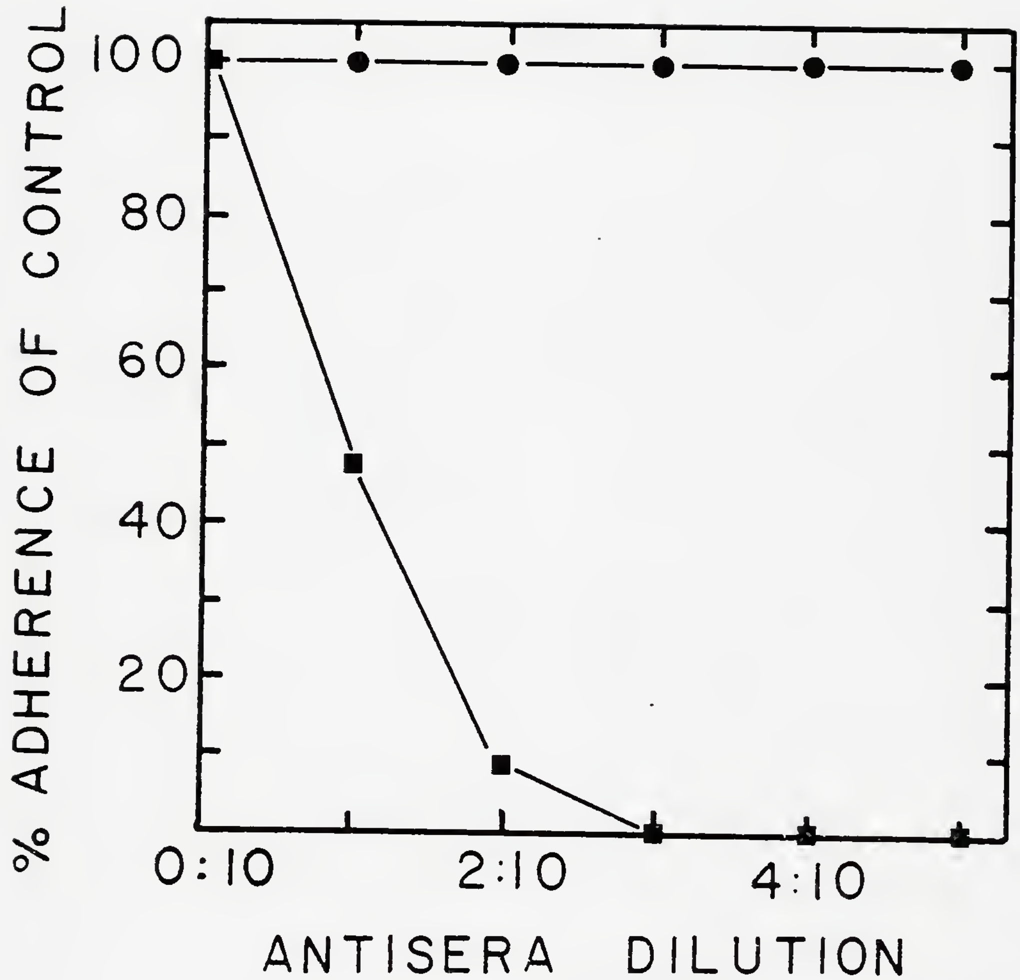

Figure 7. Titration of adherence inhibition activity of A. viscosus T14V adherence to saliva-treated HA. Normal goat serum (●); goat anti-T14V serum (■). Undiluted serum contained 133 mg protein per ml. Dilutions are expressed as ratios of antiserum volume to total volume.

Table 11

Adsorption of Goat Anti-T14V and Normal Sera

Adsorbent	% Adherence of Control[a]	
	Normal Serum	Anti-T14V Serum
None (Control)[b]	100 $\pm$ 2[c]	12 $\pm$ 1
T14V Whole Cells (7.5 mg)	87 $\pm$ 3	95 $\pm$ 3
Fibrils (7.5 mg)	107 $\pm$ 1	93 $\pm$ 1
T14V Braun Cell Walls (7.5 mg)	ND[d]	29 $\pm$ 3

[a]Serum was diluted 1:4.

[b]Controls with and without normal serum in cell-HA suspensions adhered similarly.

[c]Mean $\pm$ standard deviation.

[d]ND, Not done.

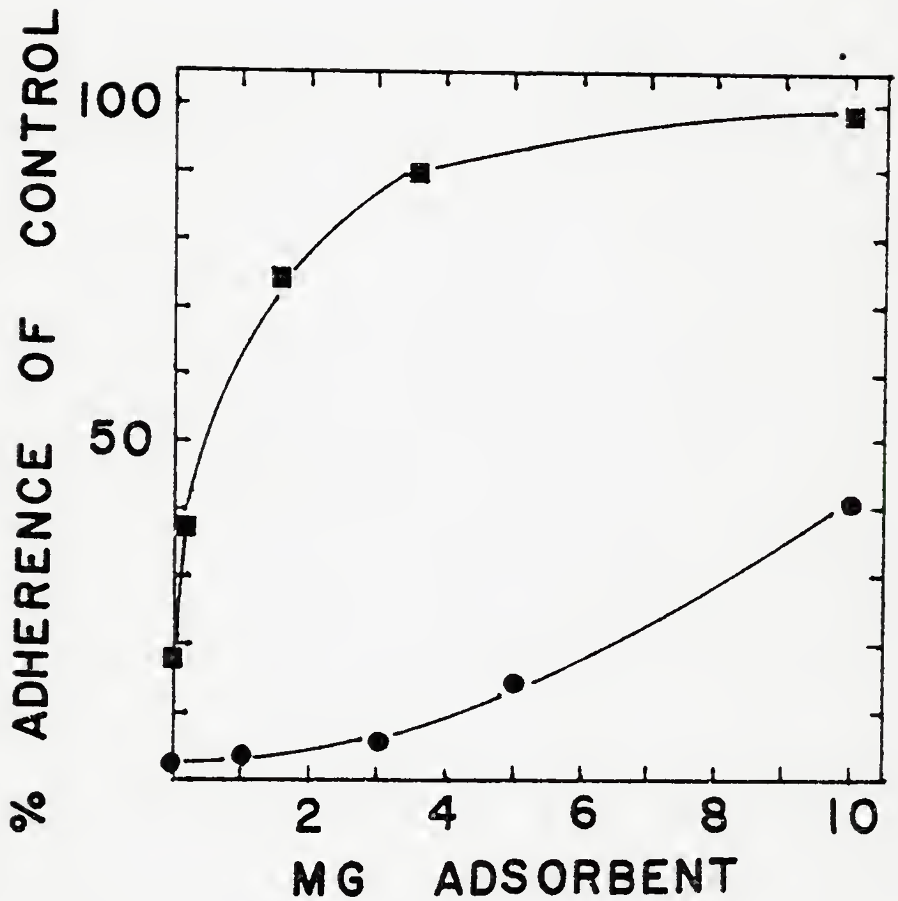

Figure 8. Adsorption of goat anti-T14V serum with A viscosus T14V whole cells (■) and Braun cell walls (●) and its subsequent effect on adherence of strain T14V to saliva-treated HA.

serum inhibition (Table 12). A volume of rabbit antifibril specific antibody equivalent to the volume of anti-T14V serum inhibited strain T14V adherence to saliva-treated HA. Undiluted rabbit anti-T14V serum contained 73 mg of total protein per ml whereas undiluted rabbit antifibril specific antibody contained 3.2 mg of total protein per ml. Adsorbed or unadsorbed normal serum did not alter adherence. In addition, inhibition of strain T14V adherence to saliva-treated HA with rabbit antifibril serum occurred at very high dilutions of the antiserum (Fig. 9).

Identification and Composition of Isolated Fibrils

Electron photomicrographs of negatively stained preparations of purified fibrils (Fig. 10) indicated the relative morphological homogeneity obtained with the fibril purification procedure. Rocket immunoelectrophoresis of purified fibril preparations demonstrated only one antigen reacting with rabbit anti-T14V serum (Fig. 11). The fibril-specific antigen was found in French press crude supernatant preparations in small amounts. The fibril-specific antigens in both preparations showed identity with one of the virulence-associated antigens (VA 1) in the whole cell Lancefield extract described previously by Powell et al. (132). In addition to the fibril-specific antigen, the crude supernatant contained two additional antigens detected by the anti-T14V serum. One of these showed identity with the other

Table 12

Influence of Rabbit Serum on *A. viscosus* T14V Adsorption to Saliva-Treated HA

Serum[a]	% Adherence of Control
Normal[b] (control)	100 ± 1[c]
Anti-T14V	10 ± 3
Antifibril Specific Antibody	9 ± 3

[a]Serum was diluted 1:4.

[b]Controls with and without normal serum in cell-HA suspensions adhered similarly.

[c]Mean $\pm$ standard deviation.

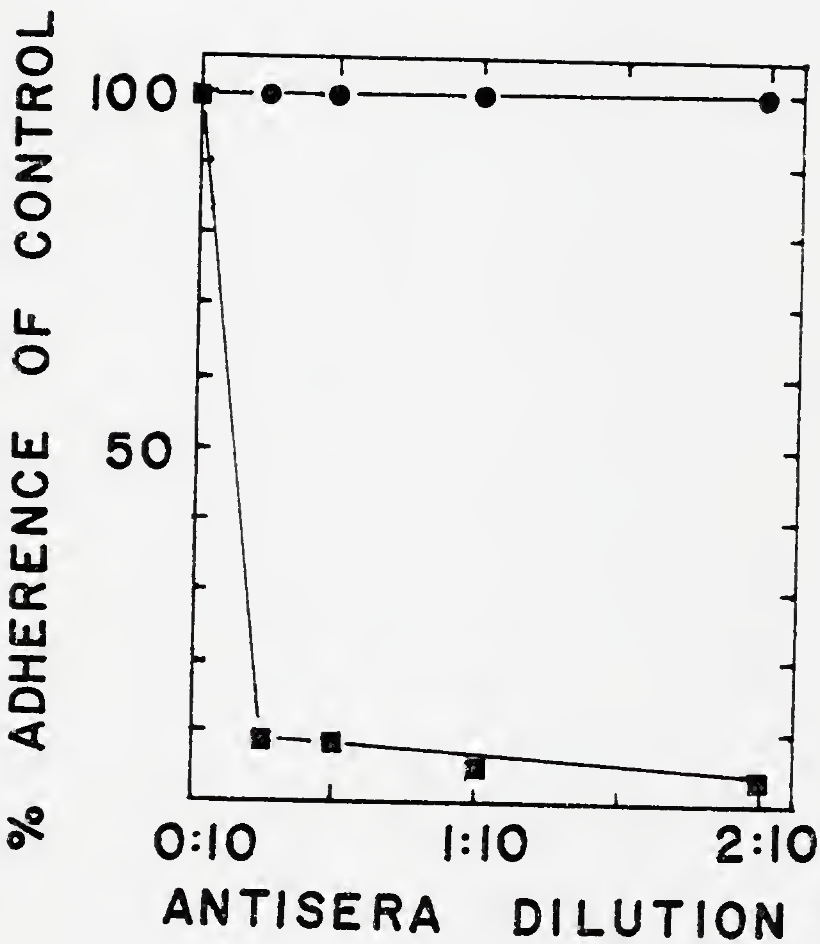

Figure 9. Titration of adherence inhibition activity of <u>A. viscosus</u> T14V adherence to saliva-treated HA. Normal rabbit serum (●); rabbit antifibril serum (■). Dilutions are expressed as ratios of antiserum volume to total volume.

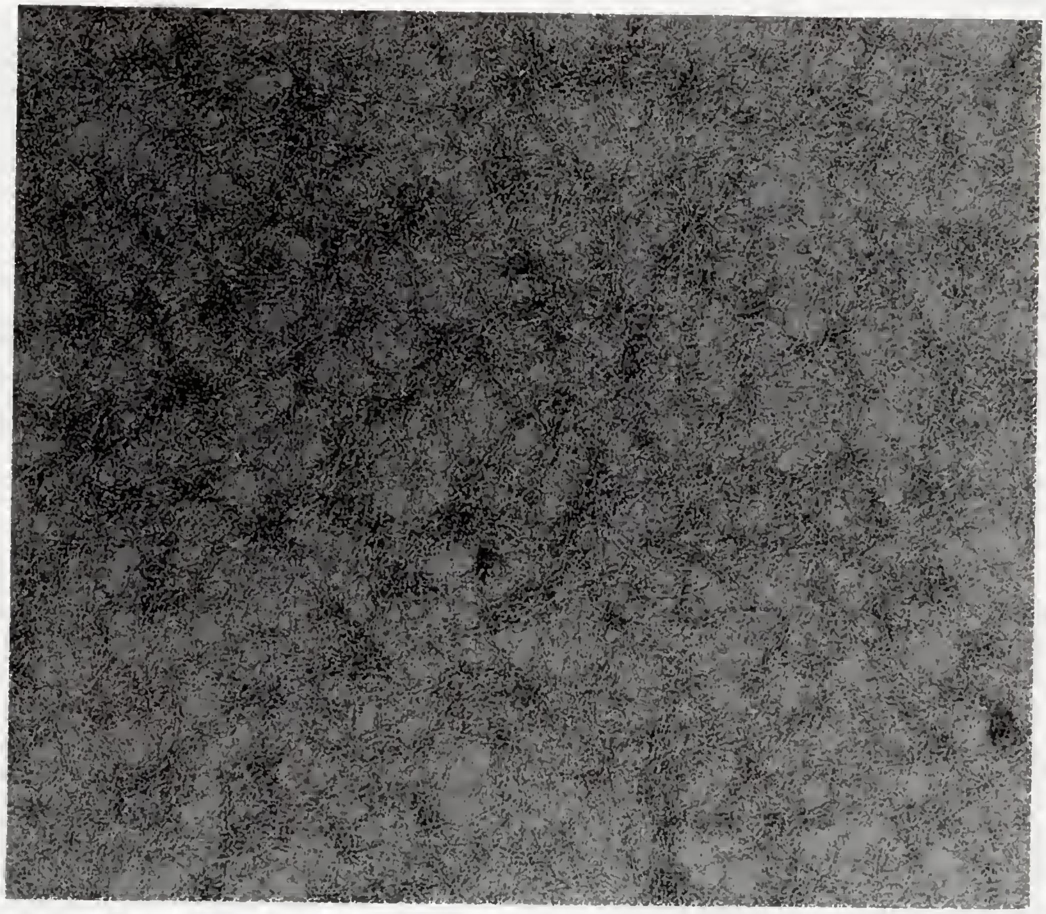

Figure 10. Transmission electron microscopy of uranyl-acetate stained preparation of A. *viscosus* T14V purified fibrils.

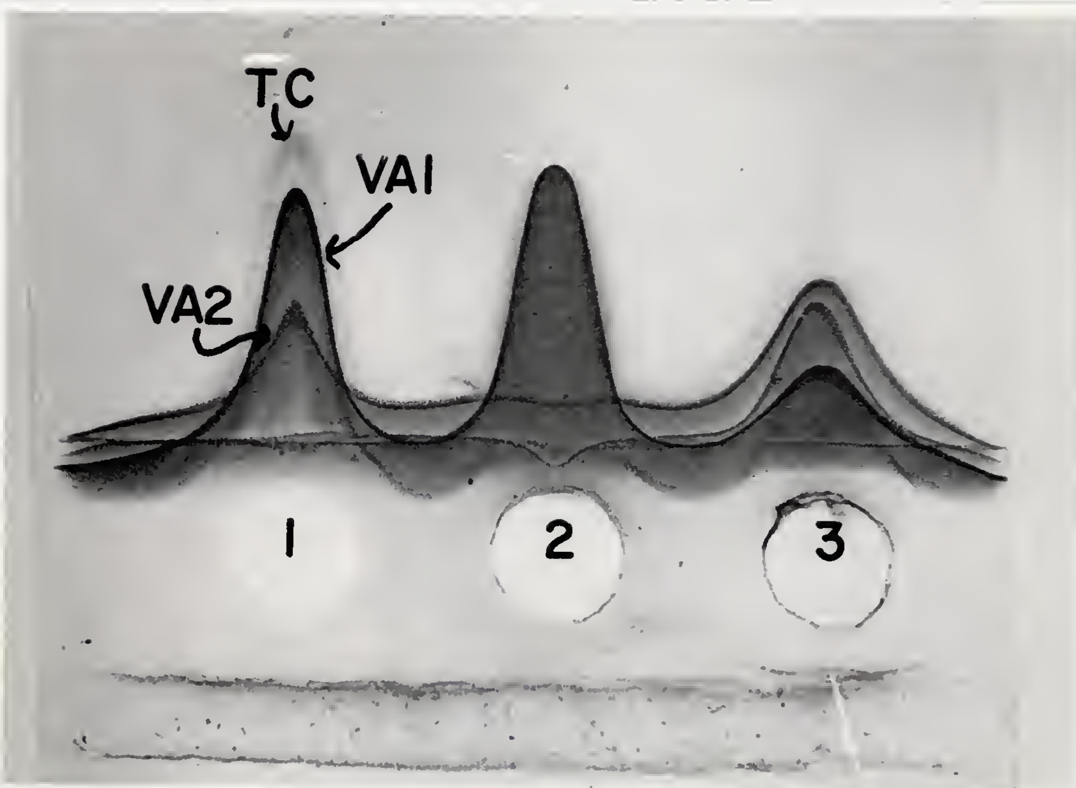

Figure 11. Laurell rocket immunoelectrophoresis of antigen preparations from A. viscosus T14V. (1) 250 µg of Lancefield extract; (2) 200 µg of purified fibrils in 0.5% Triton x-100; (3) 200 µg of French press crude supernatant. Rabbit anti-T14V whole cell serum was used at 50 µl/ml of agarose. The reference antigen in the Osserman trough was French press crude supernatant. TC, top common; VA 1 and VA 2, virulence-associated antigens 1 and 2, respectively.

virulence-associated antigen (VA 2) of the whole cell as described by Powell et al. (132). A rapidly migrating antigen was also found in the crude supernatant, but it migrated off the gel.

Immunodiffusion experiments in which crude supernatant and purified fibrils were used as antigen sources also showed a reaction of identity between one antigen in the crude supernatant and the purified fibril antigen when developed against rabbit anti-T14V serum or rabbit antifibril specific antibody (Fig. 12). In addition to the fibril-specific precipitin line, three additional precipitin lines were evident when the crude supernatant and anti-T14V serum were reacted. However, only the single precipitin line was observed in the fibril-antifibril reaction or in the crude supernatant-antifibril reaction.

Chemical analysis of purified fibrils revealed preparations composed of 95.2% protein and less than 2% carbohydrate as measured by reducing sugars. Amino acid analysis (Table 13) showed that fibrils contain high quantities of aspartic acid, threonine, glutamic acid, and alanine. Basic amino acids were present in a small quantity (15.8%) whereas acidic, polar uncharged, and nonpolar amino acids were present in approximately equal quantities (25-30%). N-acetyl muramic acid and N-acetyl glucosamine, as well as other hexosamines, were not detected in the preparation, indicating that cell wall material did not contaminate the fibril

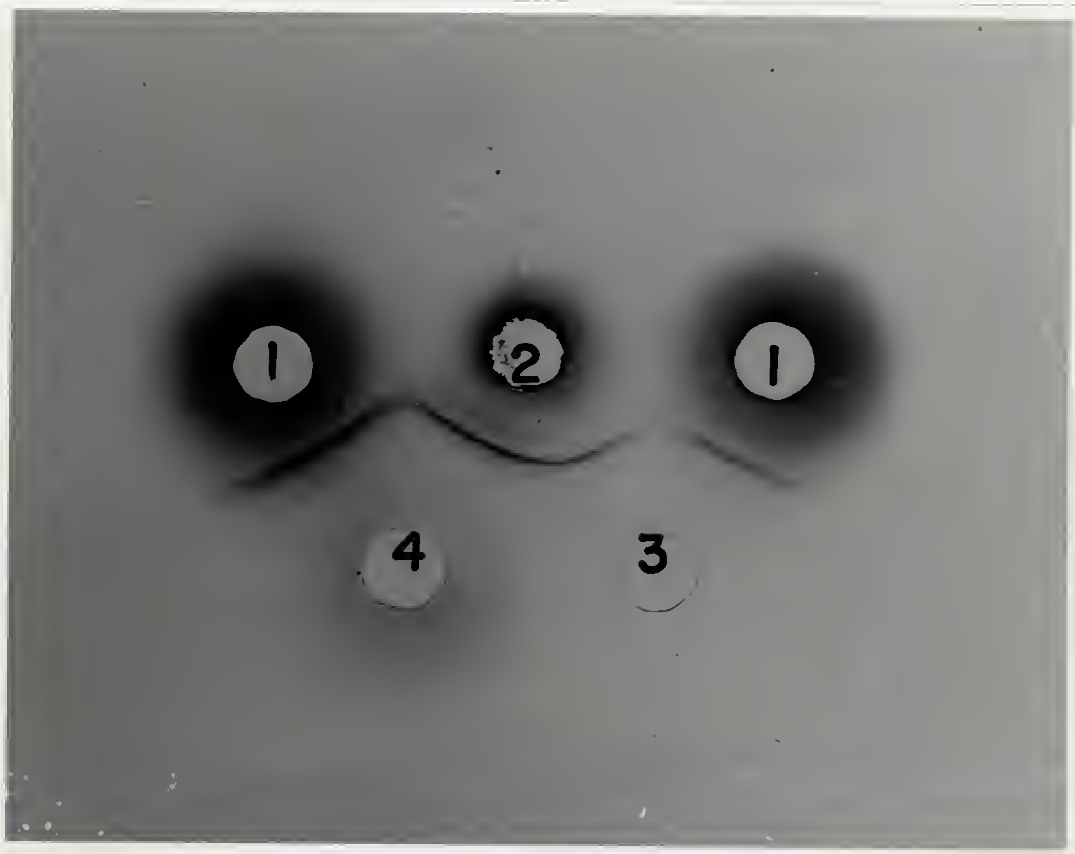

Figure 12. Immunodiffusion of antigen preparations from A. viscosus T14V. (1) 200 μg of French press crude supernatant; (2) 200 μg of purified fibrils; (3) Rabbit antifibril specific antibody; (4) Rabbit anti-T14V serum.

Table 13

Amino Acid Composition of A. *viscosus* T14V Fibrils Obtained from French Press Shearing and Lysozyme Digestion

Amino Acid	Residues per 1000[a]	
	French press	Lysozyme[b]
Acidic	(26%)	(24%)
Aspartic Acid	137	139
Glutamic Acid	120	102
Basic	(16%)	(12%)
Lysine	76	95
Arginine	70	20
Histidine	12	8
Ornithine	0	ND[c]
Polar uncharged	(29%)	(29%)
Threonine	114	136
Glycine	86	83
Serine	74	35
Tyrosine	23	33
Cystine	0	ND
Non polar	(29%)	(35%)
Alanine	102	95
Leucine	74	75
Valine	51	62
Isoleucine	28	35
Phenylalanine	23	19
Methionine	11	1
Proline	trace	62

[a]Not corrected for losses.

[b]Obtained from Cisar and Vatter (22).

[c]ND, not done.

preparation. A minimum molecular weight between 15,000-25,000 for the fibrils was calculated from the amino acid data.

Molecular sieve chromatography of isolated fibrils suspended at 1 mg per ml in phosphate buffer produced two peaks (Fig. 13). The first peak eluted at the void volume of the column and the second peak to a component corresponding to a molecular weight of approximately 12,500. The high molecular weight material was observed by TEM to contain fibrils whereas no structures resembling fibrils could be visualized by TEM from the low molecular weight fraction (data not shown). In order to determine if the aggregation of fibrils was dependent upon the fibril concentration, the concentration was decreased and then chromatographed. It was observed that the high molecular weight peak decreased; however, the low molecular weight peak actually increased (Fig. 13) suggesting that less aggregation had occurred in the dilute sample.

Molecular sieve chromatography in the presence of 8 M urea was used in an attempt to dissociate aggregates of the fibrils (Fig. 14). Two peaks were observed with the high molecular weight material eluting at the void volume and the low molecular weight material corresponding to a molecular weight of approximately 12,500. The material from these peaks was examined by IEP. Both peaks showed identity with each other as well as with the fibrils and the VA 1 antigen

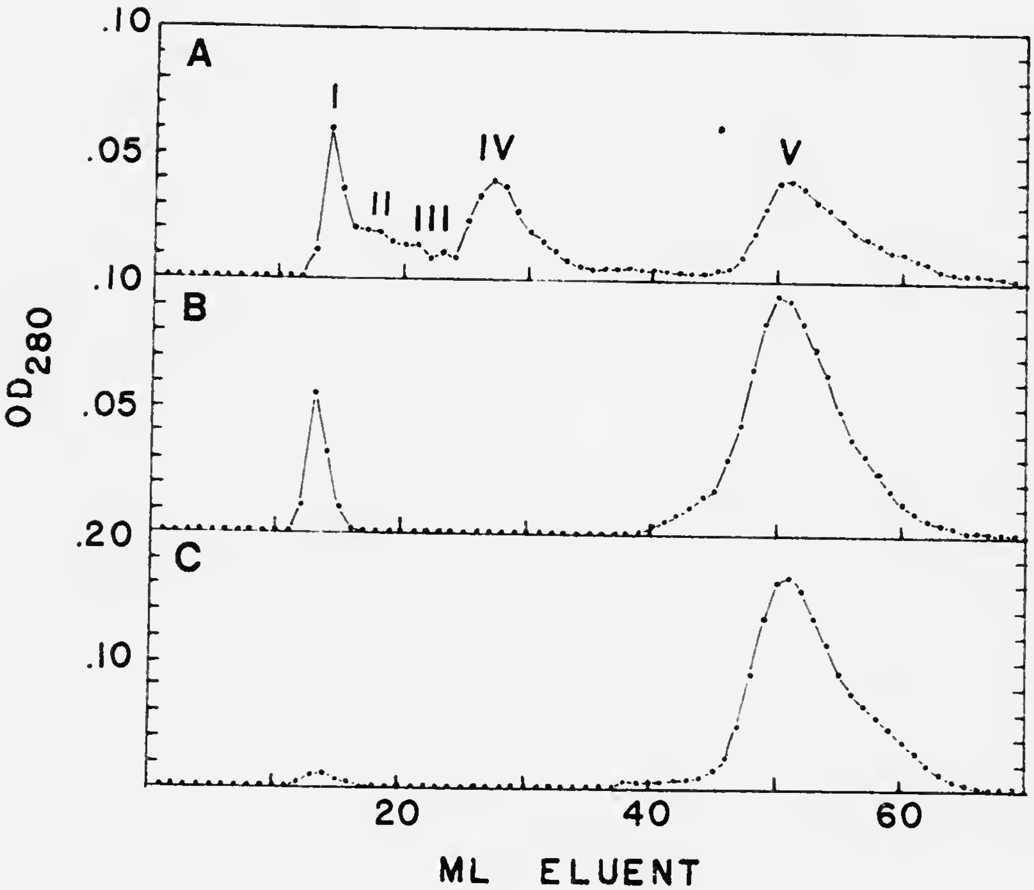

Figure 13. Molecular sieve chromatography of A) I. Blue dextran (mw 2 X 10^6); II Bovine serum albumin (mw 67,000); III Hen egg albumin (mw 45,000); IV Chymotrypsinogen A (mw 25,000); V Cytochrome C (mw 12,500); B) Isolated fibrils (1 mg/ml) and C) Isolated fibrils (0.33 mg/ml). Samples were chromatographed over an ultragel-54 column and eluted with 0.01 M potassium phosphate buffer (pH 7.2). One ml fractions were collected and absorbance at 280 nm read.

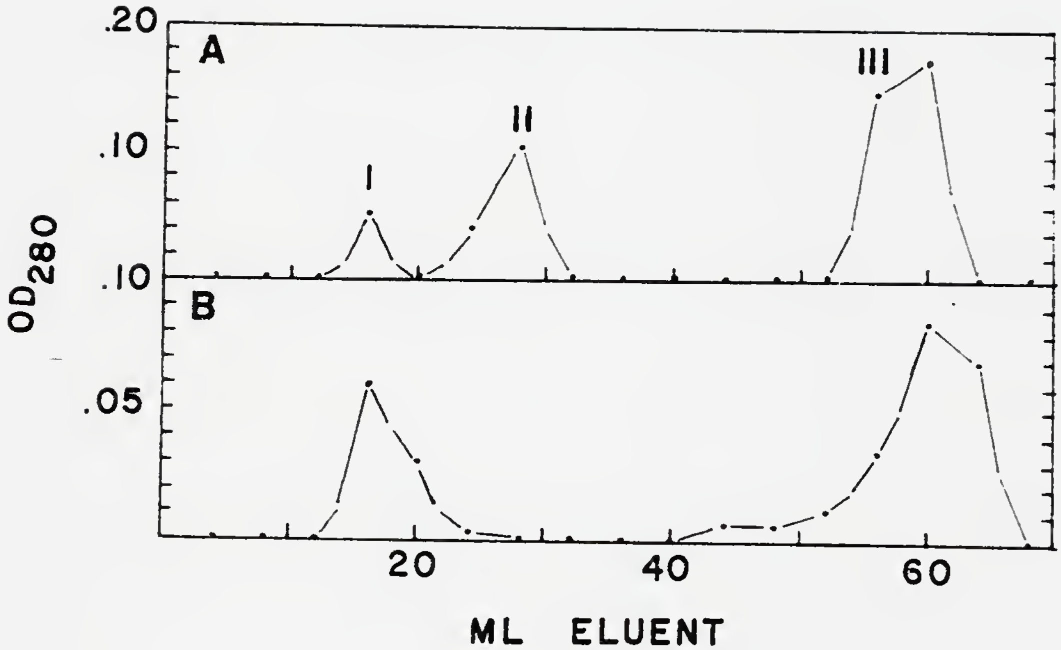

Figure 14. Molecular sieve chromatography of A) I Hen egg albumin (mw 45,000); II Chymotrypsinogen A (mw 25,000); and III Cytochrome C (mw 12,500); and B) Isolated fibrils (1 mg/ml). Samples were chromatographed over an ultragel-54 column and eluted with 0.01 M Tris buffer (pH 8.4) containing 8 M urea.

of strain T14V Lancefield extracts when the antigen preparations were subjected to electrophoresis into gel containing either anti-T14V serum (Fig. 15).

Fibril preparations subjected to PAGE-SDS produced gel patterns that contained numerous bands (Fig. 16A). The high and low molecular weight fractions obtained from molecular sieve chromatography were subjected to PAGE-SDS. The high molecular weight fraction (Fig. 16B) produced a pattern similar to the pattern produced by the fibrils. The low molecular weight fraction produced a pattern of very light bands that also was similar to the pattern of the fibrils (data not shown). However, the low molecular weight fraction did not contain the large dark band seen at the top of the PAGE gels containing the fibril or high molecular weight preparations.

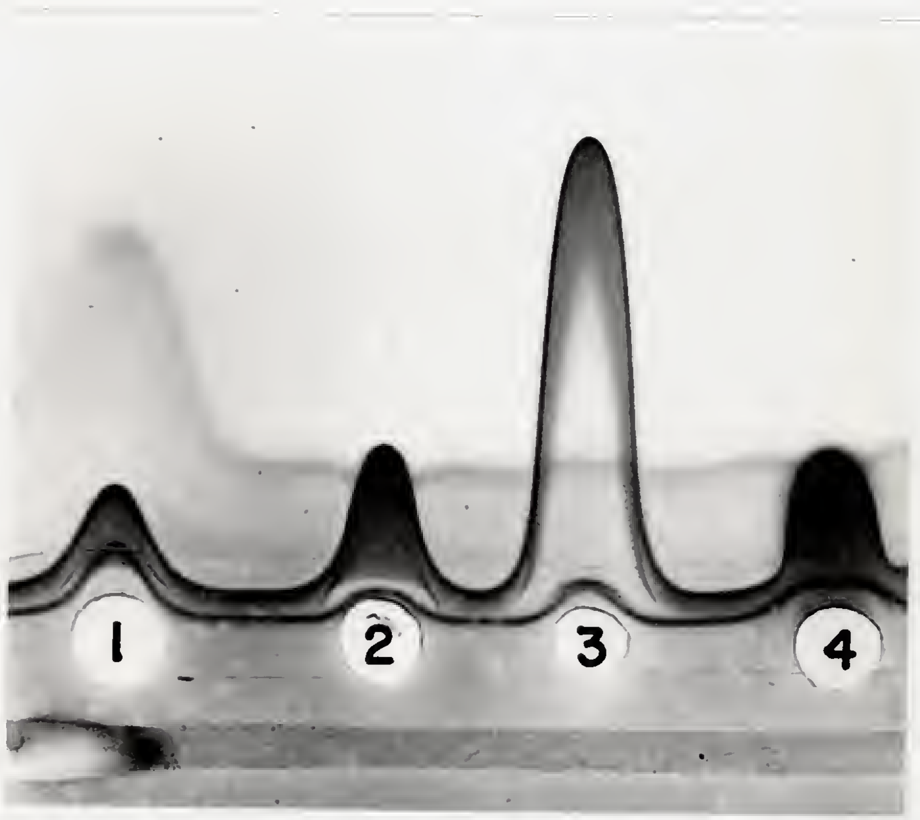

Figure 15. Laurell rocket immunoelectrophoresis of antigen preparations from A. viscosus T14V. (1) 250 µg Lancefield extract; (2) High molecular weight peak I from urea molecular sieve column; (3) Low molecular weight peak II from urea molecular sieve column; (4) 200 µg fibrils in 0.5% Triton X-100. Rabbit anti-T14V whole cell serum was used at 50 µl/ml of agarose. The reference antigen in the Osserman trough was Lancefield extract.

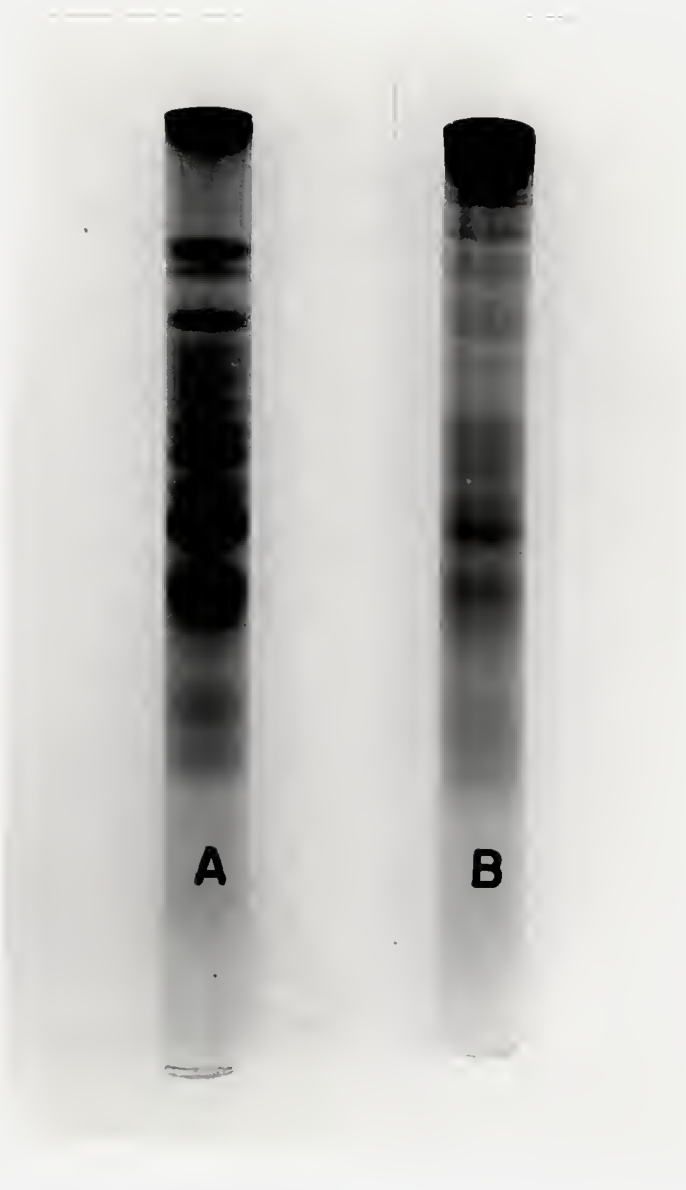

Figure 16. Polyacrylamide gel SDS electrophoresis of (A) 50 μg of purified fibrils and (B) 50 μg of high molecular weight material from molecular sieve chromatography.

DISCUSSION

Adsorption properties of the virulent *A. viscosus* strain T14V were observed to be very different from those of its avirulent variant strain T14AV. The adsorption of strain T14V to all surfaces studied appeared to follow Langmuir kinetics, as has been previously reported for other oral bacteria (1, 24, 53). The observation that adsorption isotherms of strain T14V to saliva-treated, untreated, and 20% saliva/80% serum-treated HA were generally similar indicated that adsorption to these three different surfaces followed similar kinetics. In addition, strain T14AV adsorption to untreated and saliva-treated HA also followed Langmuir kinetics, although the adsorption kinetics to saliva-treated HA was of a lower order. Strain T14AV exhibited poor adsorption to HA treated with mixtures of saliva/serum, and the data exhibited a poor fit to the adsorption model, as judged by the low correlation coefficient. Application of the adsorption model requires that a significant percentage of the cells initially available adsorb to the surface (53). Since only 6% or less of strain T14AV cells initially available adsorbed to the saliva/serum HA surface, it is not likely that these data adequately described its adsorption.

It is important to note that Langmuir kinetics are limited to monolayer adsorption to a surface (90). We have confirmed by scanning electron microscopy that a monolayer of strain T14V cells adsorbed to these HA surfaces from initial cell concentrations up to and including 4 X 10^7 cells per ml. However, at concentrations of 4 X 10^8 cells per ml and greater, cell to cell interactions were observed which lead to the formation of multiple cell layers on the HA surface. Dispersed cells of A. viscosus, A. naeslundii strains, and other oral strains are known to reaggregate rapidly (41, 58, 121, 132). Aggregation between homologous or heterologous bacterial cells has been implicated in the accumulation phases of developing plaque (58). However, the mechanisms by which cells interact with adsorbed salivary components are not necessarily the same as those involved in cell to cell aggregation (50). The series of photomicrographs (Fig. 3) illustrate that at saturating cell densities of strain T14V, cell to cell aggregation can occur. These aggregates may not be distinguishable in the in vitro adherence assay from initial attachment of the cell to the HA surface. Although the degree of cell to cell aggregation may vary from strain to strain, this possibility should not be overlooked when studying the kinetics of the initial attachment of cells to HA or other surfaces.

Calculations made from adsorption isotherms showed that HA treated with saliva or saliva/serum mixtures provided a slightly greater number of binding sites for strain

T14V than did untreated HA. Although the strength of the adsorption bond between cells of strain T14V and the saliva or saliva/serum-treated surfaces was equal to or half that calculated for the untreated HA surface, similar numbers of strain T14V cells adsorbed to untreated and saliva/serum-treated HA from fixed cell concentrations of 10^4 (data not shown) and 10^5 cells per ml. Substantially more cells adsorbed to saliva or saliva/serum-treated HA surfaces from concentrations of 10^7 cells per ml. This is consistent with a previous observation that cell adsorption at higher initial cell concentrations was more closely related to the maximum number of binding sites, whereas at low concentrations the cell adsorption was influenced by the affinity of the cell for the surface (24). Although the numbers of binding sites for strains T14V and T14AV are similar, the affinity of strain T14AV for the saliva-treated surface was 10-fold less than that calculated for strain T14V. This suggests that the feeble adsorption observed for strain T14AV is due to its relatively poor affinity for the saliva-treated HA surface. Assuming that both the virulent parent strain and the avirulent variant strain compete for similar binding sites on the saliva-treated HA surface, the adsorption model predicted that a decrease in adsorption would result from a reduced affinity between strain T14AV and the saliva surface rather than an alteration in the number of adsorption sites on the HA surface. The data support this prediction. Strain T14V also adsorbed as well to HA treated

with a mixture of 80% saliva/20% serum as to HA treated with 20% saliva in buffer, but did not adsorb well to serum or albumin-treated HA. This suggests that strain T14V cells interact specifically with adsorbed salivary components and not serum components.

It is significant that relative in vitro adsorption specificites exhibited by A. viscosus strains T14V and T14AV to saliva and saliva/serum HA surfaces were confirmed in vivo in humans by determining recoverable cell numbers from teeth after introduction of streptomycin-resistant organisms of both strains, indicating that this in vitro model mimics the adsorption specificities of human teeth in vivo. In vivo results showed that cell numbers of strain T14V averaged 10-fold higher than those for strain T14AV. In addition, the recovery of strain T14AV was less than that observed for strain T14V, indicating that strain T14AV exhibited a weaker association with the tooth surface. Thus, observations based on both the in vitro adsorption model and in vivo studies in humans indicate that strain T14AV exhibits a feeble affinity for adsorbed salivary components relative to strain T14V.

The adsorption properties of strain T14V to saliva-treated HA were very different from those of strain T14AV for the same surface when the pH of the suspending buffer was varied. The pH did not alter the adsorption of strain T14V to saliva-treated HA whereas strain T14AV adsorbed much better under acidic conditions. Several authors have proposed that plaque formation and plaque cohesion occur

via hydrogen bonding between the hydroxyl groups of bacterial polysaccharides and the acidic proteins in the salivary pellicle (78, 134). However, no direct evidence is available to support this mechanism. It has recently been reported that under specific growth conditions, strain T14AV cells produced large amounts of extracellular heteropolysaccharide and possessed a microcapsule composed of the heteropolysaccharide (14, 132). The increase in strain T14AV adsorption to saliva-treated HA in an acidic environment could be attributed to hydrogen bonding between the polysaccharide capsule and the salivary pellicle. In contrast, an acidic environment does not effect the adsorption of strain T14V to saliva-treated HA because strain T14V neither produces the large amounts of extracellular polysaccharide nor possesses a microcapsule (132). In addition, isolated strain T14AV extracellular heteropolysaccharide inhibited strain T14V adherence to saliva-treated HA (14, 131) indicating that the polysaccharide material does appear to have some affinity for the salivary pellicle.

The mechanism of bacterial adsorption to surfaces could involve electrostatic interactions. Adsorption to hydroxyapatite of polyanions such as negatively charged bacteria has been observed to be inhibited by phosphate or fluoride (137) and to be enhanced by cations such as calcium (12, 137). The negatively charged surfaces of bacteria maintain electroneutrality by sodium and potassium serving as counter ions

(135). Calcium, the most abundant divalent cation in human saliva (104), can displace the monovalent potassium and sodium ions and create a situation which favors the adsorption of bacteria to the negatively charged pellicle. Therefore, the negatively charged salivary pellicle and bacterium are bridged together by divalent calcium cations. It was observed that adsorption to saliva-treated HA by strain T14AV was increased by high concentrations of calcium as would be predicted. It has been observed that sucrose-grown S. mutans strains exhibit an increased negative charge (78, 120, 136) due to insoluble polysaccharides complexed with extracellular teichoic acid and that these sucrose-grown strains bond high amounts of calcium (78, 120). This mechanism has been used to explain, in terms of electrostatic interaction, the selective adsorption of S. mutans to teeth (79). As discussed previously, strain T14AV possesses a heteropolysaccharide microcapsule (132) which could allow for an increased negative charge and thus more complexing with calcium cations. This would result in more strain T14AV cells bound to the salivary pellicle.

Contrary to strain T14AV, strain T14V adsorption to saliva-treated HA decreased in the presence of high concentrations of calcium. Recently, it has been reported that elevated levels of calcium inhibited S. mutans adherence to saliva-treated HA (157). In addition, Fischman et al. (45) reported that cations which bind to both bacteria and the tooth surface will inhibit bacterial adsorption to teeth.

Therefore, strain T14V adsorption could be inhibited as a result of calcium binding to both the salivary pellicle on the HA and to the bacterial cell surface. The result that calcium cations could inhibit the adsorption of strain T14V to saliva-treated HA was supported by the observation that high concentrations of potassium also inhibited the adsorption. In addition, it was observed that the presence of potassium, calcium, or magnesium cations significantly reduced the adsorption of strain T14V to saliva-treated HA compared to a buffer (PBS) that did not contain any of the ions.

Since EDTA in the suspending buffer did not alter strain T14V adsorption to saliva-treated HA, calcium ions do not appear to be required. Several studies concerning the biochemistry of plaque formation have suggested that calcium cations mediate an important role in plaque formation. It has been reported that EDTA is capable of deaggregating dental palque (78). In addition, calcium ions have been shown to increase adsorption of mucins to glass and to HA (70, 115). Calcium ions appear to be required for the aggregation of both _S_. _mitis_ and _S_. _sanguis_ since the addition of EDTA inhibits aggregation (56, 76). Moreover, it has been reported that coaggregation of _A_. _viscosus_/_A_. _naeslundii_ strains with streptococcal strains (21) and hemagglutination by _A_. _viscosus_/_A_. _naeslundii_ strains (38) are dependent on calcium. However, the mechanism of _A_. _viscosus_ coaggregation

with streptococci must differ from the mechanism of strain T14V adsorption to saliva-treated HA since calcium does not appear to be necessary for adsorption to saliva-treated HA.

The use of detergents in adsorption assays produced additional data concerning electrostatic interactions of A. viscosus with the salivary pellicle. Detergents contain molecules which possess covalently linked lipophilic and hydrophilic moieties. When these agents are in solution, they concentrate at available surfaces, including bacteria, with the lipophilic portion away from the polar solvent and the hydrophilic portion toward the solvent. The ionization of the hydrophilic portion classifies these detergents into cationic, anionic and non-ionic. The non-ionic detergents carry both an anion and a cation. The adsorption of strain T14V to saliva-treated HA was not influenced by the presence of the non-ionic detergent, Tween-80. However, the anionic detergent, SDS, increased strain T14V adsorption. The reason for this is unknown; however, the SDS could increase the bacterial surface charge and provide new binding sites to which divalent cations such as calcium could then utilize to bridge the bacterium to the salivary pellicle. To answer this question, assays in SDS containing buffers both with and without calcium present, as well as EDTA, need to be done.

The cationic detergent, CTMAC, may provide the most useful and interesting detergent data. Cationic detergents have been known to possess bactericidal properties (20, 92).

Cationic detergents are also of interest because of potential reversible binding to anionic groups on oral muscosal surfaces (11, 109, 143, 153, 155) which could permit prolonged oral retention of cationic antiseptics. The quarternary ammonium compounds such as CTMAC are permanent cations due to substitution of all four hydrogens of the ammonium cation. In addition, the long lipophilic group (16 carbons) of CTMAC increases the antibacterial activity of the agent (20). Recent studies have demonstrated that several quarternary ammonium detergents, such as chlorohexidine, possess in vivo and in vitro antiplaque activities (8, 33, 62, 159, 168). In an aqueous environment such as the oral cavity, the lipophilic portion of the molecule would force these drugs to any available non-aqueous surface such as bacteria or saliva-treated HA (6). The cationic moiety would then bind the molecule to the negatively charged surfaces electrostatically. This model would fit that proposed by Rölla and Melsen (138) for the action of chlorohexidine. It was observed that the presence of CTMAC in strain T14V and saliva-treated HA suspensions completely obliterated any bacterial adsorption. However, when strain T14V cells were pretreated with the same detergent prior to their use in the adherence assay, adsorption of the organism increased. These data fit the model discussed above (6) such that the CTMAC, when included in the bacterial-HA suspension, bound to both the bacterial and saliva-treated HA surface to inhibit any adsorption. However, when the bacteria were pretreated with

the detergent, the detergent bound in a fashion that exposed the hydrophilic cationic end of the molecule and allowed the organism to adhere in greater numbers to the negatively charged pellicle. Recent reports have shown that another cationic detergent, cetyltrimethylammonium bromide, will inhibit in vitro plaque formation of A. viscosus, A. naeslundii, S. mutans and S. sanguis (165). Therefore, cationic detergents such as CTMAC may prove useful in selectively eliminating periodontopathogenic as well as cariogenic microorganisms. In contrast to strain T14V, the presence of CTMAC in the strain T14AV-HA suspension enhanced adsorption to saliva-treated HA. The reason for this is not known at this time. However, the polysaccharide capsule of strain T14AV provides a strong negative charge to the organism which may bind to the exposed cations on the HA surface.

Efforts were then directed toward further understanding the mechanism of adherence and differential affinity between strains T14V and T14AV. The observation that heat or proteolytic enzymes inhibit the adherence of both strains to saliva-treated HA suggests that the bacterial receptor molecules are associated with proteins or glycoproteins on the cell surface. Examination by TEM and negative staining of heat-treated cells which did not adsorb well to HA revealed that fibrils had been destroyed by the treatment. Recent reports have also shown that TSB cultures of strain T14AV possess fewer surface fibrils than those of strain T14V (23, 132). The number of cell surface receptor

molecules which interact with salivary binding sites could be expected to influence the affinity the cell has for the surface. This could explain the low affinity which was observed with strain T14AV for saliva-treated HA and human teeth. It was of interest that 0.2% chymotrypsin had little effect on the adherence of strain T14V cells to saliva-treated HA but abolished the adherence of strain T14AV. However, an increase in the concentration to 1.0% chymotrypsin did dramatically inhibit the adherence of strain T14V cells. This observation suggests that strain T14V may possess quantitatively more enzyme-sensitive binding material (i.e., fibrils) than did strain T14AV. Alternatively, its binding material might be less available for enzymatic digestion. McIntire and co-workers (116) and Cisar and co-workers (21) have suggested that the receptor site for cell to cell aggregation between _A. viscosus_ strain T14V and certain _S. sanguis_ strains also may be associated with the surface fibrils.

It has been suggested that specific bacterial surface components such as the fibrils of _A. viscosus_ may interact in a specific "lectin-like" manner with components comprising the acquired pellicle, thereby accounting for the specificity of bacterial attachment to teeth (43, 53, 55, 93). Cisar et al. (21) reported that coaggregation of actinomycetes and streptococci was not only calcium dependent and heat sensitive, but that coaggregation was reversible in the presence of lactose. It was proposed that the

"lectin-like" receptor responsible for the coaggregation was specific for lactose (116). It was observed that neither lactose nor other common carbohydrates would inhibit strain T14V adsorption to saliva-treated HA. This suggests that either another sugar not tested may be the receptor molecule, that the receptor molecule may involve a sidechain of a specific combination of various sugars, or that the lectin mechanism is not applicable.

A variety of studies have shown that specific surface polymers possessed by various bacteria may interact with host tissue components (19, 30, 39, 44, 65, 69, 122, 139, 161) facilitating firm attachment to a specific host tissue. For example, the adherence of *Klebsiella pneumoniae* to rat bladder epithelial cells was recently shown to be inhibited by antiserum to the whole cells and by specific antiserum to the surface pili (44). In addition, Salit and Gotschlich (139) reported that antipilus sera inhibited adherence of piliated strains of *Escherichia coli* to monkey kidney cells. In the present study the adherence of *A. viscosus* strain T14V to saliva-treated HA was shown to be mediated by surface fibrils. Adherence was completely inhibited by both goat and rabbit anti-T14V and antifibril rabbit serum. Adherence inhibition activity could be adsorbed from the antiserum with strain T14V whole cells and with purified fibrils immobilized on Sepharose beads. Antifibril specific antibody eluted from immobilized fibrils also inhibited the adherence of strain T14V cells to saliva-treated HA.

The adherence inhibition activity decreased significantly as anti-T14V serum was preadsorbed with increasing quantities of strain T14V cells. It is of interest that for preadsorption of the antiserum, cell walls were less effective than whole cells in eliminating adherence inhibition activity. For example, when 4 mg of either whole cells or cell walls was used as adsorbent, inhibition by whole cell-adsorbed serum decreased by more than 50% whereas inhibition by cell wall-adsorbed serum decreased less than 10%. Since whole cells have additional mass within the cells, it seems logical to expect that adherence inhibition would decrease to a greater extent if specific antisera were preadsorbed with cell walls rather than an equivalent weight of whole cells. However, this was not observed. In addition, Powell (131) has observed recently that strain T14V cell walls neither adhere to saliva-treated HA nor possess the long surface fibrils characteristic of whole cells.

Cisar and co-workers (22, 23) isolated fibrils from strain T14V cell walls that had been digested with lysozyme. Furthermore, antibody that reacted with these isolated fibrils also reacted with intact fibrils on the outer surfaces of strain T14V whole cells (23). The Braun procedure for obtaining cell walls removes the cell surface fibrilar material (131). However, it is possible that short fragments remain imbedded in the cell wall matrix and are available for binding antibody directed against the fibrils. Subsequent treatment of the cell walls with lysozyme

apparently releases the fibril fragments isolated by Cisar and Vatter (22). Initial adsorption of a cell to a surface is thought to entail a reversible phase, whereby at equilibrium the cell remains approximately 100 Å from the surface, balanced between the repulsive effects of negatively charged surfaces and the attractive influences of Van der Waal's forces (107). Thus, it is possible that the fibril fragments that remain in the cell walls after Braun homogenization are not of sufficient length to bridge this 100 Å gap and permit walls to adhere firmly to saliva-treated HA. Considering the apparent similarity in morphology and antibody reactivity of the surface fibrils and matrix fibrils observed by Cisar and Vatter (22) and by us, it is not surprising that the amino acid composition of the fibrils isolated by the method described here was similar to the composition obtained by lysozyme digestion of cell walls (Table 3) (22). Collectively, these data suggest that fibrils isolated by shearing whole cells may be representative surface extensions of the entire fibrils, which were isolated from enzyme digested walls by Cisar and Vatter (22).

Fibrils were also included in bacterial cell-HA mixtures in order to determine whether strain T14V adherence could be competitively inhibited. Addition of fibrils or crude supernatant, which contains other cell surface components in addition to fibrils, to a suspension of strain T14V cells and saliva-treated HA decreased adherence by as much as 30%. This suggests that purified fibrils compete for the

strain T14V binding sites on the saliva-treated HA, thereby blocking cellular adherence at those sites. Fibrils are also thought to mediate cell to cell aggregation of strain T14V cells (131). Therefore, fibrils adsorbed to receptor sites on a saliva-treated HA surface could also act as alternate adsorption sites for strain T14V cells. Such alternate sites which mask adsorbed salivary receptor sites could promote a less effective attachment, causing adherence inhibition. This is diagrametically depicted in Figure 16. As discussed previously, strain T14V adhered as a monolayer of cells to saliva-treated HA at initial cell concentrations of 4 X 10^7 cells per ml (Fig. 17A). When the initial cell concentration was increased to 4 X 10^9 cells per ml, cell to cell interactions lead to the formation of multiple cell layers on the saliva-treated HA surface (Fig. 17B). If fibrils mediated the adherence of strain T14V to specific salivary receptors, it would be anticipated that the incubation of fibrils with saliva-treated HA would result in the fibrils adsorbing to the salivary receptors (Fig. 17C). If fibrils were included in bacterial cell-HA suspensions, fibrils adsorbed to the salivary receptors would inhibit whole cells from adhering to those specific receptors. However, if the fibrils also mediated cell to cell aggregation, the fibrils adsorbed to the salivary receptor sites could act as secondary receptors for the strain T14V cells (Fig. 17D).

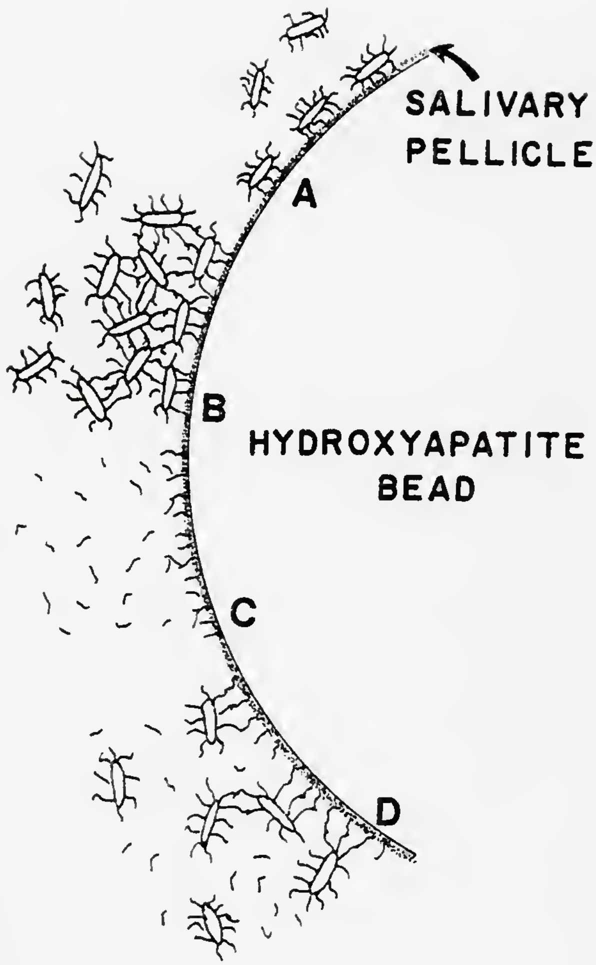

Figure 17. Diagramatic representation of strain T14V (A) monolayer formation and (B) multiple cell layer formation; (C) saturation of specific salivary sites with fibrils; and (D) strain T14V cells adsorbing to the fibrils.

Inhibition was increased to 47% by decreasing the number of strain T14V cells in the assay 100-fold. The parameters that control an organism's adsorption to a surface are the affinity of the organism for that particular surface and the number of receptors that particular surface has available for the organism. By decreasing the number of strain T14V cells available for binding and thus eliminating any bacterial saturation of available salivary receptors, the organism's affinity for the surface controls its adsorption to the salivary receptors. Therefore, if the affinity of strain T14V for the fibril receptors is less than the affinity of strain T14V for the salivary receptors, a higher precent inhibition would be expected to occur. The data obtained support this hypothesis.

Additional evidence that strain T14V affinity for salivary receptors was greater than the affinity for fibril receptors has been provided by two observations. First, it was observed that strain T14V cells aggregated when suspended in whole clarified saliva (unpublished observation). However, if HA was included in the strain T14V-saliva suspension, the bacteria adsorbed to the HA surface and aggregation did not occur (unpublished observation). This indicated that the strain T14V cell had a higher affinity for salivary receptors on HA than for aggregating receptors. Secondly, adsorption isotherms determined by optical density of strain T14V cell concentration up to and including 10^{10} cells per ml, revealed the formation of a biphasic isotherm

curve (data not shown). The first phase of the curve appeared identical to the cell to surface adsorption discussed previously. The second phase of the curve represented cell to cell interactions resulting in the formation of multiple cell layers observed by SEM. This indicated that the affinity of the strain T14V was greater for the saliva-treated HA than for receptor molecules on the cell surface.

Characterization of the fibrils was begun by using a variety of techniques. The fibrils appeared as a homogenous suspension as judged by TEM. Antigenic homogeneity of the fibrils was shown by immunodiffusion and Laurell rocket IEP. Laurell rocket IEP further identified a single fibril antigen showing identity with the VA 1 antigen previously found in Lancefield extracts of strain T14V by Powell et al. (132). A second antigen in the crude supernatant showed identity with the VA 2 antigen of Powell et al. in the strain T14V Lancefield extract (132). The VA 2 antigen of Lancefield extracts was previously believed to be a result of the extraction procedure which altered the VA antigen to form two antigenic moieties (23). However, this does not appear to be the case since crude supernatants that had not been Lancefield extracted also contained both antigens. In addition, Lancefield extraction of fibrils did not alter the immunoelectrophoretic pattern (data not shown).

Chemical analysis of the fibrils revealed that the preparation was greater than 95% protein. Carbohydrate content,

determined as reducing sugars, showed the fibril preparations to be void of any such carbohydrates. Amino acid analysis provided data that revealed the fibrils did not contain any amino-sugars.

A minimum molecular weight ranging from 15,000-25,000 for the fibrils was calculated from the amino acid analysis data. Attempts were made to clarify the molecular weight by: 1) column chromatography in the presence of potassium phosphate buffer, 8 M urea in tris (hydroxymethyl) aminomethane buffer at pH 8.4, and 8 M were in glycine buffer at pH 3.0; 2) polyacrylamide gel electrophoresis in a non-reducing cationic gel system (PAGE), a SDS-reducing gel system (PAGE-SDS), and a 8 M urea-reducing gel system (PAGE-urea). Fibrils chromatographed on the various columns produced an absorbance 280 peak at the void volume as well as at a molecular weight corresponding to approximately 12,500. The findings that 1) the void volume fraction was viewed under TEM to contain fibrils, 2) the two column fractions showed identity with one another by IEP, and 3) the low molecular weight peak could be increased by decreasing the fibril concentration loaded onto the column, led us to speculate that the fibrils found in the void volume fraction consisted of aggregated fibrils whereas the low molecular weight fraction contained the subunit or monomer form.

Solubilization of the purified fibril preparations was a prime problem in their characterization. Fibrils were not

completely soluble in phosphate buffers, SDS, urea, or Triton X-100. A non-reducing PAGE gel of fibrils revealed a single dense band at the top of the gel (data not shown). Suspension of the fibrils in 8 M urea caused solubilization as observed visually; however, column chromatography and PAGE-urea of fibril preparations revealed two absorbance 280 peaks and numerous coomassie blue stained bands, respectively (data not shown). Fibril preparations and the high and low molecular weight fractions obtained by column chromatography produced similar gel patterns in PAGE-SDS systems. The observation that the low molecular weight fraction PAGE-SDS gel did not contain the dense band found at the top of the high molecular weight and fibril gels could indicate that the band consists of aggregated fibrils. This observation further supports the hypothesis that the high molecular weight fraction consisted of aggregated fibrils whereas the low molecular weight fraction contained monomer forms. Recently it has been reported that the common type 1 pili of Escherichia coli are resistant to solubilization by SDS (117) and 6 M urea (139) and resistant to hydrolysis by trypsin (117). Studies with a protein isolated from Brassica juncea revealed that SDS at low concentrations caused aggregation and the PAGE-SDS gels revealed numerous bands (133). However, increased concentrations of SDS caused the protein to dissociate and eventually one band was observed in PAGE-SDS gels. The glycoprotein, thyroid stimulating hormone, was not completely soluble in SDS, urea, or propionic acid (156).

Concentrations of SDS as high as 5% caused only 25-30% dissociation of the hormone. Urea concentrations up to 7.1 M increased dissociation of the hormone up to 70%. Hormone treated by both these solvents produced numerous bands in PAGE-SDS systems. Dissociation of the hormone was increased to 90% by treatment with propionic acid. Experiments to attempt to increase the fibril solubility must be done. Higher concentrations of SDS and propionic acid are various treatments that will be used. When a high percentage of dissociation is finally achieved, PAGE-SDS gels will allow an estimation of the molecular weight of the subunit composing the fibrils.

The results of this investigation can be summarized as follows. *A. viscosus* strain T14V adhered to saliva-treated HA in vitro to a higher degree than did strain T14AV. This differential in adherence was determined to be due to the lower affinity of T14AV for the in vitro salivary pellicle. Serum was used to form a pellicle that would mimic the pellicle found in the gingival crevice. The decrease in adsorption of strain T14AV to this surface was determined to be due to the further decrease in the affinity of the cell for the salivary pellicle. Therefore, the virulence attributed to strain T14V over strain T14AV is a result of the affinity for the salivary pellicle.

In vivo experiments in humans supported the results obtained from in vitro experiments. That is, higher numbers of strain T14V cells than strain T14AV cells were recovered

at various times from subjects that were inoculated with equivalent numbers of both strains.

Experiments designed to study the role, if any, of hydrogen and/or ionic bonding in the adsorption of both strains yielded interesting results. Strain T14AV adsorption involved hydrogen and electrostatic bonding possibly between the polysaccharide capsule with the salivary pellicle. Adsorption of strain T14V to the salivary pellicle apparently is not significantly influenced by either hydrogen or ionic bonding.

The adsorption of strain T14V to saliva-treated HA was sensitive to heat as well as to proteolytic enzymes which is characteristic of a protein receptor molecule. The strain T14V adsorption could be inhibited by antiserum to the whole cell or to the extracellular surface fibrils. In addition, the antisera inhibition activities could be removed by adsorbtion of sera with strain T14V cells or purified fibrils. This provides evidence that the fibrils are at least one mediator of strain T14V adherence for saliva-treated HA.

Theoretically, local antibodies directed against the bacterial cell surface receptor(s) which promotes binding to the host tissue might be expected to exert some modulating influences on the initial adherence and subsequent colonization. For example, cell associated glucosyltransferase is known to enhance the accumulation of S. mutans in plaque on the teeth of conventional rats (25, 169). It has been shown that immunization with purified glucosyltransferase

effectively reduces colonization by S. mutans and protects against the subsequent development of dental caries in conventional and gnotobiotic rats (166) and hamsters (146). Thus, the isolation and identification of surface structures responsible for the adherence of A. viscosus strain T14V cells to salivatreated HA surfaces and the inhibition of strain T14V adherence by antifibril specific antibody suggest the possibility that these surface fibrils might be effective for inducing immunoprotection possibly interfering with colonization by this organism. The apparent role of fibrils in the in vitro adherence of strain T14V cells to rat saliva-treated HA and in the in vivo colonization of rats by this organism has been suggested by Brecher et al. (14). The rodent model offers a reasonable in vivo model testing whether colonization by strain T14V can be controlled by artificially inducing immunoprotection.

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Timothy Thomas Wheeler was born on March 24, 1954, in Buffalo, New York, where he lived for 14 years until moving to Fort Lauderdale, Florida, in 1968. He attended Stetson University where he received a Bachelor of Science degree in 1976. On May 29, 1976, he married the former Eliza F. Nyad of Fort Lauderdale, Florida. In September, 1976, he entered graduate school in the Department of Immunology and Medical Microbiology, College of Medicine, University of Florida. On September 26, 1978, the birth of his son, Timothy T., Jr., occurred. He is author of two major publications and six abstracts. Upon completion of his graduate studies in June, 1980, he will become a post-doctoral fellow in the Department of Basic Dental Sciences, College of Dentistry, University of Florida. He plans to enter dental school in September, 1981, and then pursue a career in the field of basic science and clinical dental research.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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